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DIRECTORATE OF DISTANCE EDUCATION

M.Sc

II-SEMESTER

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BIOPROCESS TECHNOLOGY

SYLLABI-BOOK MAPPING TABLE

BIOPROCESS TECHNOLOGY

Syllabi	Mapping in Book
UNIT I An overview of fermentation technology Overview of fermentation technology Range of fermentation processes Primary and secondary metabolites Components of fermentation process	**Pages 1 - 17**
UNIT II Industrial Microorganisms Industrial microorganisms, isolation and preservation of Industrial important organisms, screening, strain improvemen and maintenance.	**Pages 18 - 69**
UNIT III Industrial Media formulations Formulation of industrial media – Medium Requirements for fermentation processes, carbon, nitrogen, mineral sources, buffers, antifoam agents, and medium optimization.	**Pages 70 - 94**
UNIT IV Microbial Growth Kinetics Stoichiometry of cell growth and product fermentation, Sterilization of media and fermenters, Scale-up process and Strater culture technology..	**Pages 95–126**
UNIT V Basic Design of a fermenter-I Basic design of a microbial fermenter, types of fermentation vessels, aseptic operation and containments.	**Pages 127 -15274**
UNIT VI Basic Design of a fermenter -II Body construction (stirrer glands, bearing, valves, steam traps) baffles, spargers and impellors.	**Pages 153-184**
UNIT VII Design of a Fermenter -III Types of fermentation – batch, continuous, fed-batch, solid state and submerged fermentation.	**Pages 185 -208**
UNIT VIII Fermentation methods Aerobic and anaerobic, duel and multiple fermentations, their advantages and disadvantages.	**Pages 209 - 219**
UNIT IX Down Stream Processing-I Importance of downstream processing in industrial fermentation process, Problems ad requirements of bio product recovery and purification.	**Pages 220 - 228**

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UNIT -I: FERMENTATION TECHNOLOGY

Structure

1.1 INTRODUCTION

After going through this chapter you will be able to know, what is fermentation? What are the basic requirements of industrial fermentation? Also, the types of materials are industrially produced. Further, it covers the role of microorganisms and the basics of fermeters.

1.2 OBJECTIVES

After going through the chapter you will be able to;

- Understand the concept of fermentation.
- Basic requirements of the fermentation.
- Understanding the microbial process.
- Know the primary and secondary metabolites.
- Know the needs of fermenter.

1.3 OVERVIEW OF FERMENTATION TECHNOLOGY

The term “Fermentation” is derived from Latin Verb “Fervere” means to boil. Fermentation is the process involving the biochemical activity of organisms, during their growth, reproduction, even senescence and death. Naturally, microorganisms use sugar or starchy material and to convert in to acids or alcohols. During the process of fermentation

observe the formation of gas bubbles (CO_2↑). The term "fermentation" was applied to the process in which alcohol was formed from sugar. Pasteur described "fermentation is an anaerobic process through which microorganism obtained energy for growth in the absence of oxygen". Recently, fermentation is defined as "both aerobic & anaerobic metabolic activity of microorganism in which specific biochemical changes are brought in an organic substrate. Fermentation technology is defined as the use of organisms to produce food, pharmaceuticals and alcoholic beverages on a large scale industrial basis. The basic principle involved in the industrial fermentation technology is that organisms are grown under suitable conditions, by providing raw materials meeting all the necessary requirements such as carbon, nitrogen, salts, trace elements and vitamins. Examples of the microbial fermentation include bread, cheese, wine, vinegar, ethanol beer, enzymes, amino acids, antibiotics, hormones, compost, biopolymers, bioplastics, microbial oils, flavors, colorants, specialty chemicals, vaccines, therapeutic proteins, and numerous other useful products.

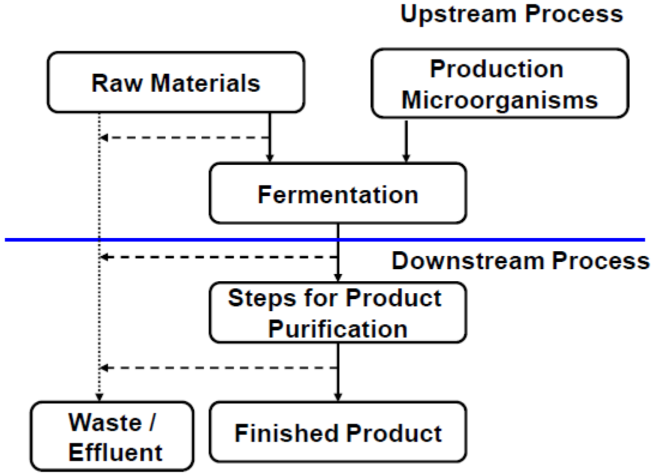


Fig-1: Industrial processing.

Commonly, fermentation is initiated by inoculating a suitable substrate with the desired microorganism. Under the favorable environmental condition the microorganism converts the substrate to the desired product. The fermented crude product may be used directly, or it may be processed (purified) further to isolate specific metabolite from it.

Successful development of a microbial fermentation process requires the multidisciplinary contributions, particularly biochemistry,

chemistry, genetics and molecular biology, chemical and process engineering, and mathematics for statistical modeling and computer technology. A typical process engages both upstream (USP) and downstream (DSP) processing stages (Fig. 1). The upstream process is associated with all factors that are essential for the fermentation. It consists of three main divisions. They are,

The producer microorganism: The key factor that responsible for product fermentation, obtaining the microorganisms includes, the strategy for initial screening of suitable industrial microorganism, strain improvement to enhance productivity and yield, pure culture maintenance of the strain, preparation of a suitable inoculum and fermentation conditions to improve the economic efficiency of the process. For example, the production of stable mutant strains that greatly overproduce the expected metabolite is often crucial. Some microbial products are primary metabolites, produced during active vegetative growth (growth phase or trophophase), for example; amino acids, organic acids, vitamins and industrial solvents such as alcohols and acetone. However, many valuable industrial products are secondary metabolites, they are not necessary for microbial growth, for example; antibiotics and alkaloids, usually at after optimum biomass production (stationary phase growth or the idiophase).

The fermentation medium: The selection of suitable economic culture medium enriched with low cost carbon and energy sources along with other essential nutrients are important for higher production of the industrially valuable products. In this, optimization of the media components is a vital aspect to ensure maximization of yield and economics. In many of the industrial media, the basis raw materials are waste products from other industrial processes, particularly sugar processing wastes, corn steep liquor, lignocellulosic wastes, cheese and whey.

The fermentation process: Industrial fermentation process is normally under strictly controlled conditions developed to optimize the growth of the microorganism or production of a target microbial product. The synthesis of microbial metabolites is usually tightly regulated by the microbial cell. In order to obtain high yields, the physiological (environmental) conditions that trigger regulatory mechanisms, is suitable for product formation, in particular, it should not repress or feedback control, must be avoided.

Industrial fermentations are performed in large size culture vessels called fermenters, often with capacities of several thousand litres. Fermenters may be stirred or unstirred, to complex integrated systems involving varying levels of computer control or automation. The fermenter must be constructed basically by stainless steel, that can be repeatedly sterilized and that will not react unfavorably with the microorganisms or with the target products. The mode of fermentation process either batch or, fed-batch or continuous, the system of its aeration and agitation, where necessary, all these approaches are taken to process scale-up.

Conventional downstream processing (DSP) includes all individual processes that follow up of fermentation. The major process involve in DSP are cell harvesting, cell disruption, product purification from cell extracts or the growth medium (supernatant/filtrate), and end with finishing steps. However, recent days all the process is being made to join together in fermentation with DSP operations. It increases process productivity. Overall process of product recovery should be rapid, efficient and also maintain the maximum purity of the product with stability. The stability of the product is important, if it is unstable in impure form or mixed with other contaminants, is not suitable for safe uses. For example, enzymes need maximum purity; otherwise it loses its activity during storage. Other than that, there must be safer and inexpensive disposal of waste products generated during the process is important.

1.4 FERMENTATION METHODS

There are three different types of fermentation; they are (1) Batch fermentation, (2) Feb-batch fermentation and (3) Continuous culture. Batch fermentation is defined as the fermentation process is carried out in defined volume at a particular period of time. In batch fermentation six phases of the microbial growth [lag phase, acceleration phase, log phase, deceleration phase, stationary and death] is observed. In fed-batch fermentation, freshly prepared culture media is added at regular intervals without removing the spent culture fluid. This increases the volume of the fermentation culture. This type of fermentation is used for production of proteins from recombinant microorganisms. However, in the continuous fermentation the products are removed

continuously along with the cells and the same is replenished with the cell growth and addition of fresh culture media and it maintain steady state microbial growth. This type of fermentation is used for the production of single cell protein (SCP), antibiotics and organic solvents.

Microbial fermentation technology can be grouped into four major categories such as 1) Microbial biomass production (SCP); 2) Microbial metabolites [a) Primary metabolites (ethanol, citric, acid, glutamic acid, lactic acid, acetic acid, acetone, formic acid, butanol, propionic acid, dihydroxy-acetone, glycerol etc) and b) Secondary metabolites (antibiotics, alkaloids, toxic pigments, vitamins etc)]; and Microbial Enzymes (Glucose oxidase, protease glucoamylase, amylase, glucose isomerase, rennin, pectinase, superoxide dismutase, cellulase, invertase, lactase and lipase). In some time, the fermenting microbes have got the capacity to convert an added substrate into some more valuable product, ex. conversion of ethanol to acetic acid (vinegar), isopropanol to acetone, glucose to gluconic acid, sorbitol to sorbose (this is used in the manufacture of vitamin C), sterols to steroids. Among all these bioconversions, the production of steroids is the most widely applied fermentation biotechnology for the conversion of sterols into steroids, like cortisone, hydroxycortisone, prednisone, dexamethasone, testosterone, estradiol etc.

Further, the fermented metabolites or the products are recovered and separated by a sequence of processing called downstream processing. There are various methods used for downstream processing. In this, first of all, the broth is conditioned i.e. the cells are aggregated and form large clumps, which makes the separation easier. The conditioning is done by heating, freezing, pH change, antigen-antibody reactions etc. Then the conditioned broth is used for the separation of the constituents for which techniques like sedimentation, floatation, filtration, ultra-filtration, centrifugation and micro-filtration are applied.

1.5 THE RANGE OF FERMENTATION PROCESS

Microbial fermentation Processes can be classify into five different categories, they are

1. Microbial Enzymes

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2. Microbial Metabolites
3. Microbial Cells (Biomass) as the Products
4. Recombinant Products
5. Modifying Substrates (Transformation Process)

There are several sources available for the production of industrially valuable enzymes, which includes microbes, plants and animal. However, commercial productions of many enzymes in industries are by the use of microorganisms. As being produced in large quantities by the fermentation processes, microbial enzymes have the enormous economic potential. Moreover, microbes are more prone to change in its genetics to enhance its productivity compared to plant or animal system. Through the genetic engineering and also the metabolic engineering, our desirable eukaryotes enzyme is possible to produce in the prokaryote systems. In addition, it is possible to control and improve microbial enzyme production by introducing inducers and activators in the production medium.

Enzymes such as amylases, cellulases, pectineses, proteases, lipases have industrial applications. Amylases, beta gluconases and cellulases have the applications such as mashing (Crushing, Squashing, Smashing), manufacture of syrup, preparation of precooked baby foods, breakfast foods, manufacture of soft centre candies and also for backing industries. Industrially proteases have the applications such as improvement of dough texture, reduction of mixing time, and increase in loaf volume, chill proofing for wine preparation, production of whole milk concentrates, ice-cream & frozen desserts, and curdling milk in food industries, dehairing and baiting for leather industries, detergents preparation and also for recovery of silver from spent film. Pharmaceutically valuable anti-blood clotting streptokinases are produced from bacterial sources. As like proteases, lipases also used for textile industries and pharmaceutical industries

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Table-1: Industrial usable enzymes and its applications.

Enzyme	Source	Industrial application
Amylase	Fungal/ bacterial	Reduction of dough viscosity, acceleration of fermentation, increase in loaf volume, improvement of crumb softness, and maintenance of freshness Baking and milling Mashing for brewing Precooked baby foods, breakfast foods of cereals
	Bacterial	Improvement of dough texture, reduction of mixing time, increase in loaf volume Manufacture of syrups for prepare Chocolate and cocoa Manufacture of high-maltose syrups from corn Desizing of fabrics for textile detergents
Protease	Fungal/ bacterial	Chill proofing for brewing Manufacture of protein hydrolysates , and Stabilization of evaporated milk, curdling milk in dairy industry Detergents In laundry Dehairing, baiting in Leather process industries Tenderization of meat, protein hydrolysate
β-Glucanase	Fungal/ bacterial	Improvement of fine filtration
Pectinase	Fungal	Coffee bean fermentation
Pectinase, hemicellulase	Fungal	Preparation of coffee concentrates
Invertase, pectinase	Fungal/ bacterial	Manufacture of soft center candies and Low temperature processing for Confectionery
	Fungal	Clarification of fruit juices
Amyloglycosi dase	Fungal	Production of glucose from corn syrup from corn
Glucose isomerase	Fungal	Manufacture of fructose syrups from corn
Lactase	Fungal / Bacterial	Production of whole milk concentrates, ice cream, and frozen desserts, Removal of wood waxes. Detergents
Glucose oxidase	Fungal	Glucose removal from Eggs; Oxygen removal and Soft drinks stabilizers
Streptokinase	Bacterial	Antiblood clotting

Check your Progress-1
Note: Write your answer in the space given below
a. List the types of fermentation methods.
b. Define fermentation.
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1.6 MICROBIAL METABOLITES

Microbes can able to produce different economically valuable metabolites. Microbial metabolites are generally classified into primary metabolites and secondary metabolites. After the fresh inoculation of a culture into fermentation medium microbes starts to multiply exponentially and it reach maximum. In this phase is usually called growth phase, microbes utilize complex organic substances to produce a simple metabolites (anabolites) essential for its growth (include amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, etc). They are called primary metabolites which they are produced (equivalent to the log, or exponential phase) as the trophophase. After it reaches a constant maximum growth it eventually ceases the growth and the cells enter the so-called stationary phase and also the death phase.

During the period of trophophase, there are various metabolic products which together constitute the primary metabolites. The primary metabolites are further divided into two categories they are; 1) Primary essential metabolites, and 2) Primary metabolic end products.

1. **Primary essential metabolites**: These are the important compounds requires to maintain the process of cell growth, and hence these are produced in enough quantity. Vitamins, nucleosides, amino acids are the example of the essential metabolites.

2. **Primary metabolic end products:** Compounds such as ethanol, butanol, acetone, and lactic acid are the normal end products of the fermentation process of primary metabolism. Although these products are not important, sometimes have industrial importance too. For instance, carbon dioxide (CO_2) is the metabolic end-product of the *Saccharomyces cerevisiae*.

Importance of the primary metabolites

Many products of primary metabolism are of considerable economic importance and are being produced by fermentation [Table 2]. The synthesis of primary metabolites by wild microorganisms is such that their production is satisfactory to meet the requirements of the organism. Hence, the task of the industrial microbiologist is to modify the wild-type strain to improve the productivity of these compounds. This has been attained very productively, over several years, by the range of induced mutants, the use of recombinant DNA technology (rDNA), and the control of the fermentation conditions of the producing organism. This is demonstrated by the production of amino acids where productivity has been increased by several fold.

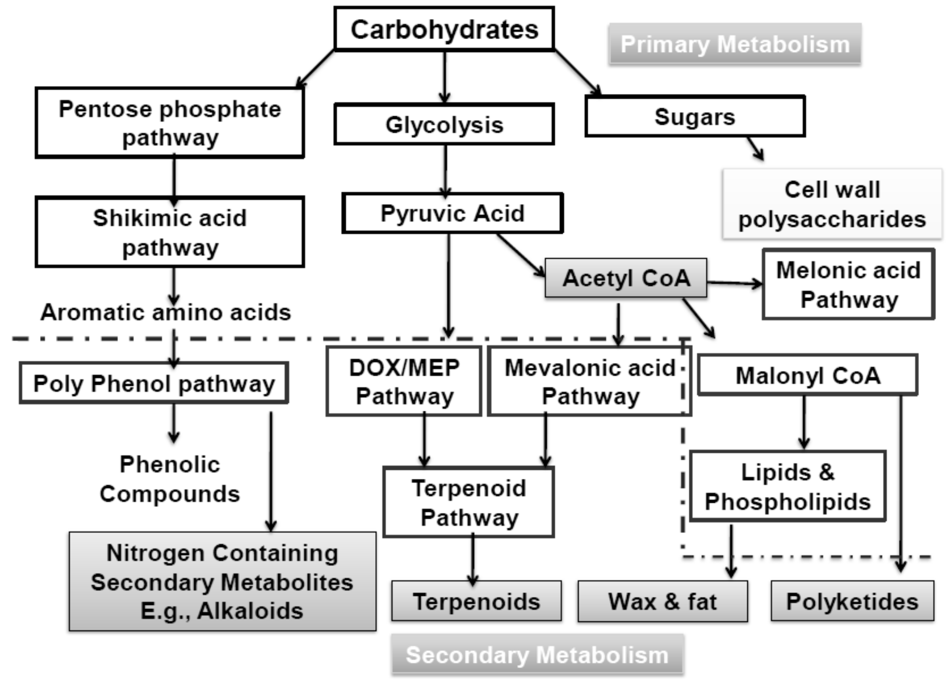


Figure – 2: Primary and secondary metabolic activity of the cell.

In recent years, the advances in metabolic engineering arising from genomics, proteomics, and metabolomics have provided new modern techniques to further understand the physiology of "over-production" and to engineer the microorganisms to "over-produce" of primary metabolites.

Table -2: Primary metabolites and its applications.

Metabolite	Application
Lysine	Feed supplement
Phenylalanine	Precursor of aspartame, sweetener
Polysaccharides	Applications in the food industry Enhanced oil recovery
Ethanol	"Active ingredient" in alcoholic beverages Used as a motor-car fuel when blended with petroleum
Organic acids	Various uses in the food industry
Glutamic acid	Flavor enhancer
Vitamins	Feed supplements
Nucleotides	Flavor enhancers

Secondary Metabolites

After the trophophase or the exponential phase ends, the process enters into the phase called as the deceleration and stationary phases or otherwise known as idiophase or secondary metabolism phase**.** In this period some microbial cultures synthesize compounds which are not produced during the trophophase. That compounds which do not appear to have any noticeable function in cell metabolism are referred to as the secondary compounds (idiolites). It is important to understand that secondary metabolism may occur in continuous cultures at low growth rates and is a property of either slow-growing or non-growing cells, where the microorganisms grow at relatively low growth rates in their natural environment. This phase occurs at the period of the limited nutrient or when there is the accumulation of the waste products.

Table-3: Secondary metabolites and its applications.

Secondary Metabolite	Applications
Penicillin, cephalosporin, streptomycin	Antibiotics
Avermectins	Antiparasitic agents
Cyclosporine A	Immunosuppressant
Bleomycin, mitomycin	Anticancer agents
Lovastatin	Cholesterol-lowering agent
Taxol	Anticancer

The interrelationships between primary and secondary metabolism are illustrated in Fig-2, and the differences of the secondary metabolites and the primary metabolites are given in the Table-4.

Table-4: Differences of Primary and Secondary metabolites

Primary Metabolites	Secondary Metabolites
1. The metabolism products that are produced during the growth phase of an organisms in order to perform the physiological functions and supports in overall development of the cell are called primary metabolites.	1. The end products of primary metabolism that are synthesized after the growth phase has been completed and are important in ecological and other activities of the cell are known as secondary metabolites.
2. Trophophase	2. Idiophase
3. Growth phase	3. Stationary phase
4. These are produced in large quantities, and their extraction is easy.	4. These are produced in small quantities, and their extraction is difficult.
5. Same in every species, which means they produce the same products.	5. Varies in different species.
6. These products are used in industries for various purpose.	6. Secondary metabolites such as antibiotics, gibberellins are also important.
7. Primary products play the significant role in the cell growth, reproduction and development.	7. They also indirectly support the cell, in sustaining their life for long duration.
E.g., Vitamins, carbohydrates, proteins and lipids are some of the examples.	E.g., Phenolics, steroids, essential oils, alkaloids, steroids are few examples.

Mostly, primary metabolic root is common to all microorganisms; however, the secondary metabolic activity of the organism is entirely different from other microorganisms. The secondary metabolism exhibited by a very wide range of different microorganisms. Also, not all microorganisms undergo secondary metabolism. Many secondary metabolites have antimicrobial activity, others are specific enzyme inhibitors, some are growth promoters and many have pharmacological

properties. The physiological function of secondary metabolism in the producer organism in its natural environment has been the issue of extensive debate and their functions include effecting differentiation, inhibiting competitors, and modulating host physiology. Thus, the taxonomic distribution of secondary metabolism is fairly different from that of primary metabolism. It is vital to understand that the classification of microbial products into primary and secondary metabolites is a suitable one. The compounds like antibiotics, alkaloids, steroids, gibberellins, toxins, etc. do not have the direct relation to the synthesis of cell materials and their growth, but are produced in small quantity. Hence secondary metabolites are considered as the end products of the primary metabolites.

1.7 MICROBIAL CELL BIOMASS

Three types of microorganism are used for microbial biomass production; they are bacteria, fungi (including higher fungi) and microalgae. Preferred characteristics of the microorganisms are having high specific growth rate, high substrate to biomass yield, high cell density, ability to use complex substrates, affinity to substrate, and low nutrient requirements. The desired metabolic products are extracted and the remaining biomass is either regarded as waste or further processed using, for example, anaerobic digestion. In the case of microalgae, the metabolic products such as lipids are chemically transformed route similar to refinery operations to produce biodiesel or lead chemicals. Biofuels produced in this way are referred to as fourth generation biofuels. On the other hand, natural poly-microbial consortia have many characteristics that are desirable in many biotechnological processes.

In 1996, new sources mainly biomasses of yeast, fungi, bacteria and algae are named Single Cell Protein (SCP) as coined to describe the protein production, originating from different microbial sources. Microbial biomass has been considered an alternative to conventional sources of food or feed. Large-scale processes for SCP production show interesting features, including:

- The wide range of methodologies, raw materials and microorganisms are used for the production of biomasses.
- High efficiency in substrate conversion
- High yield, resultant from the fast growth rate of microorganisms
- Independence of seasonal factors

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In this category, yeast was the first microorganism used as animal feed supplement. At present, several technologies have been reported to reduce the Nucleic Acid content of microbial cells, including both chemical and enzymatic procedures. Various microorganisms used for the production of SCP are algae (*Spirulina*, *Chlorella*, etc.), molds (*Trichoderma*, *Fusarium*, *Rhizopus*, etc.), bacteria (*Cellulomonas*, *Alcaligenes*, etc.), and yeast (*Candida*, *Saccharomyces*, etc.). For the production of biomass, microorganisms can utilize a variety of substrate like agricultural wastes, industrial effluents, solid and semisolid waste, natural gas like methane, etc. Few important microbial biomass as SCP are listed in Table-5.

Table-5: Microbial SCP and raw materials for SCP production.

Microbial SCP	Raw material for Biomass production
Aeromonas hydrophylla	Lactose
Aeromonas delvacvate	n-Alkanes
Acinetobcter calcoacenticus	Ethanol
Bacillus subtilis, Flavobacterium sp.	Cellulose, hemicellulose
Lactobacillus sp.	Glucose, Maltose
Methylomonas methylotrophicus	methanol
Pseudomonas fluorescens	Non-protein sources
Rhodopseudomonas capsulate	Glucose
Candida tropicalis	Glucose, Maltose
Candida utilis	Glucose
Candida novellas	n-alkanes
Candida intermedia	Lactose
Saccharomyces cereviciae	Lactose, pentos, maltose
Aspergillus fumicatous	Maltose, Glucose
Penicillium cyclopium	Lactose, Galactose
Rhizopus chinensis	Glucose, Maltose
Tricoderma viridae	Cellulose, Pentose
Spirulina sp., Carbondioxide, Porphyrium sp., Chlorella sp., Nostoc sp., Scnedesmus sp.	Carbon dioxide for photosynthesis, Industrial effluents, Glucose

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1.8 RECOMBINANT PRODUCTS

The advancements in genetic engineering and recombinant DNA technology have extended the range of possible fermentation products. Genes from higher organisms (any eukaryotes) may be introduced into microbial cells as recipients, are capable of synthesizing “foreign” proteins and desirable metabolites. These proteins are described as “heterologous”, it means derived from a different organism. A wide range of microbial cells has been used as hosts for such systems including *Escherichia coli*, *Saccharomyces cerevisiae*, and filamentous fungi. Products produced by such genetically modified organisms (GMOs) include interferon, insulin, human serum albumin, factors VIII and IX, epidermal growth factor, calf chymosin, rennet, and bovine somatostatin. Important factors in the design of these processes include maximizing the expression of the foreign gene, the larger scale secretion of the product, minimization of the product degradation, and control of the onset of synthesis during the fermentation.

Table-6: Recombinant microbial products.

Product	Purpose
Alpha-glucosidase	Pompe’s disease
Interleukin-4 receptor	Asthma
TNF-α	Rheumatoid arthritis
Vascular endothelial growth factor	Cardiovascular disease
HIV vaccine	AIDS
Neurex	Cystic fibrosis
Insulin	Diabetic

Transformation Processes

Transformation is a process of simple modification on the chemically complex feed stock chemicals in to usable compounds. Microbial cells may be used to convert a compound into a structurally related, financially more valuable, compound. Microbial enzymes can behave as chiral catalysts with high positional specificity and stereospecificity, also highly specific than pure chemicals. It enables the addition, removal, or modification of functional groups at specific sites on a complex molecule without the use of chemical protection. The reactions, which may be catalyzed, include oxidation, hydroxylation, dehydrogenation, dehydration, decarboxylation,

deamination, isomerization and condensation. In addition, microbial processes exhibit relatively low temperatures, pressures and without the requirement for potentially polluting heavy-metal catalysts. For example, the production of vinegar (conversion of ethanol to acetic acid) is the oldest microbial transformation process. These processes involve the production of high-value compounds including steroids, antibiotics, and prostaglandins.

Table-7: Microbial transformation products.

Substrate	**Product**	**Microorganism used**
Acetonitrile	Acrylamide	*Rhodococcus rhodochrous*
Ethanol	Acetic acid	*Acetobacter sp.*
Cholic acid	Ketocholic acids	*Alcaligenes faecalis*
Progesterone	1,4-androstadiene-3,17-dione	*Fusarium solani*
21-acetoxy-3β-hydroxy-5-pregnen 20-one	21-hydroxy-4-pregnene-3- 20-dione	*Corynebacterium mediolanum*
Oxandrolone	90-Hydroxyoxandrolone	*Rhizopus stolonifer*
Testosterone	7β-Hydroxytestosterone	*Chaetomium sp.*
Hydrocartisone	11 β-Hydroxyandrost-4en-3,17-dione	*Neurospora crassa*

1.9 COMPONENT OF FERMENTATION PROCESS

In spite of the type of fermentation a conventional process may be divided into six basic component or parts, they are:

1. The formulation of fermentation media to be used in the fermentation process during the development of the inoculum and in the production fermenter.
2. The method and equipments required for sterilization of the medium, fermenters, and ancillary equipment.
3. The production of an active, pure culture in sufficient quantity to inoculate the production vessel.
4. The growth of the organism in the production fermenter under optimum conditions (physiological factors such as aeration, agitation, heat transfer, pH, Temperature, osmotic pressure) for product formation.

5. The extraction of the product and its purification.
6. The disposal of effluents produced by the process.

The interrelationships between the above six components are described in Fig. 3. However, one must also visualize the research and development program which is designed to gradually improve the overall efficiency of the fermentation. Before a fermentation process is established a producer organism has to be isolated, modified such that it produces the desired product in commercial quantities, its cultural requirements determined and the plant designed accordingly. Also, the extraction process has to be established. The development program would involve the continual improvement of the process organism, the culture medium, and the extraction process. The details of the process are described in the following chapters.

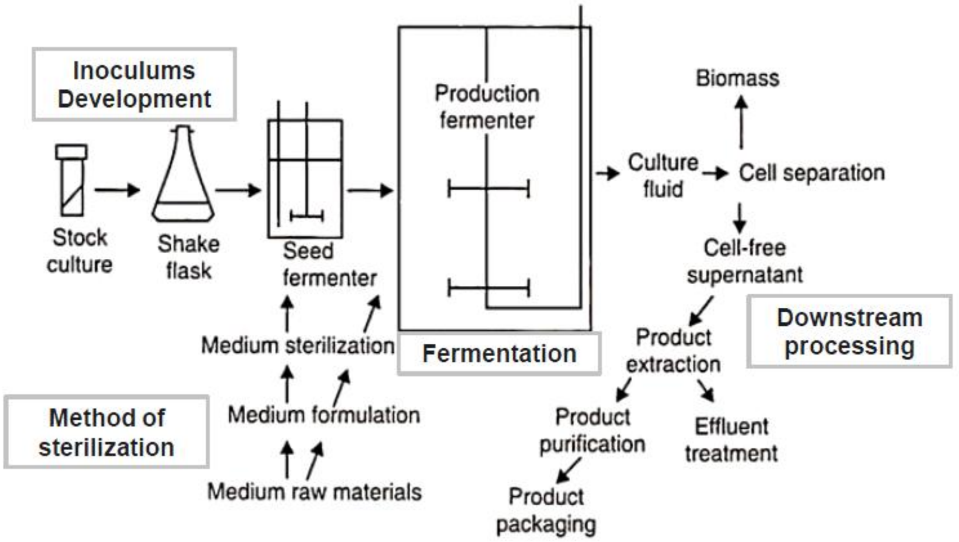


Figure - 3: Components of fermentation process.

Check your Progress -2
Note: Write your answer in the space given below
c. What are primary metabolites?
d. Give two examples for recombinant microbial products.
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1.10 LET US SUM UP

In this chapter, you have learnt about the meaning, definition, need, objectives, importance and concept of microbial fermentation.

This knowledge would make you understand what fermentation is and how it can be used for human being. The concept such as microbial metabolites, primary and secondary metabolites, and its commercial importance would have made you to distinguish them and their functional activities. The methods of fermentation, aerobic and anaerobic, batch, continuous and fed batch process with submerged and surface fermentation pattern have clearly illustrated. The recombinant microbial products and its value are clearly described. This content might play very important role in your studies.

1.11 UNIT - END EXERCISES

1. Differentiae primary metabolites from secondary metabolites.
2. Explain the various methods of industrial fermentation.
3. Describe the role of microbes in bioprocess technology.

1.12 ANSWERS TO CHECK YOUR PROGRESS

a. Batch fermentation, fed-batch fermentation and continuous fermentation.
b. Fermentation is defined as “both aerobic & anaerobic metabolic activity of microorganism in which specific biochemical changes are brought in an organic substrate.
c. Primary metabolites are the substrates or metabolic intermediates essential for the normal growth of the organisms. E.g. Glyceroldehyde 3-P, Citric acid, amino acids.
d. Insulin for diabetics, TNF-α for Rheumatoid arthritis.

1.13 SUGGESTED READINGS

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UNIT -2: INDUSTRIAL MICROORGANISMS

Structure

2.1 INTRODUCTION

After going through this chapter you will be able to;

- Understand the concept of sources of industrial microbes
- Know the methods of primary screening, importance of secondary screening, highthroughput screening.
- Understands the basic concepts for strain improvement methods, mutant analysis, and maintenance of the industrially important microbes.
- Value the role of microbes in economically important industrial fermentation.

1.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the concept of screening methods
- Relate the important of strain improvement- genetic engineering method.
- Know the techniques of culture maintenance.
- Importance of high throughput screening.
- Know the culture collection centers.

2.3 SCREENING OF INDUSTRIALLY IMPORTANT MICROORGANISMS

The microorganism with extraordinary biochemical characters has been used for production of commercially valuable products in fermentation industries. Industrial microorganisms have been isolated from the natural environments such as soil, air and water, also in the extreme environments. The period in between 1950s and 1960s was called as “golden era of antibiotic discovery”, where the soil microorganisms are screening their products, yielded the major groups of currently use antibiotics. However, some lacuna has been faced in the period of late 1980s and 1990s; hence the pharmaceutical industries took major policy decision to cease the previous activity and construct new methodologies for screening of antibiotic producing organisms as well as other industrially usable metabolite producers. Thus, the basic principles of strain isolation will be considered to be important for the origin of many current industrial producers.

The first step in the screening of potential microorganisms with industrial application is the isolation. Isolation means “obtaining either pure or mixed cultures with desired characters or to produce the desired product”. Some industrial organisms may be isolated directly by a specific design of the protocol named screening methods. It may be a single step process or multistep procedures. However, it should be more economic, reaction dependent, and safe selection criteria. The selection criteria as being important in the choice of organism:

1. The nutritional characteristics of the organism.
2. Depending on the value of the product, a process may have to be carried out using a predetermined cheap medium.
3. The optimum temperature of the organism.
4. The cross reaction of the organism with the equipment to be used for the fermentation and the type of process to be used.
5. The stability of the organism and its suitability to genetic manipulation.
6. The productivity of the organism, ability to convert substrate into product and to give a high yield of product per unit time.
7. The ease of product recovery from the culture.

If an organism contains some specific feature to produce toxic metabolites or may react with the equipment used for its production/

recovery process should be properly assessed. Hence the basic characters of the producing microorganism must be required for the natural isolates or otherwise, the isolates may be obtained from any of the culture collection centers (Table- 1). Such collections may provide organisms of known characteristics with satisfactory information's for the product formation. In addition, it is certainly cheaper than that culture may be isolated from nature, but it is also true that a superior organism may be found after an exhaustive search of a range of natural environments. Further, designing suitable model systems to develop the culture and assay techniques that may be required to assess the quality of natural isolates.

Table-1: Major Culture Collections Centres

Culture Collection	
1.	National Collection of Type Cultures (NCTC)
2.	National Collection of Industrial Food and Marine Bacteria (NCIMB Ltd)
3.	National Collection of Yeast Cultures (NCYC)
4.	UK National Collection of Fungus Cultures
5.	The British Antarctic Survey Culture Collection
6.	National Collection of Plant Pathogenic Bacteria
7.	American Type Culture Collection (ATCC)
8.	Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ)
9.	Centraalbureau voor Schimmelcultures (CBS)
10.	Czech Collection of Microorganisms (CCM)
11.	Collection National de Cultures de Microorganismes (CNCM)
12.	Japan Collection of Microorganisms (JCM)

The ideal isolation procedure originates with an environmental source (soil) which is highly suitable to be rich in the desired properties, is used. Selective pressure may be used in the isolation of organisms that will grow on particular substrates. However, if it is not possible to apply selective pressure for the desired character, it may be possible to design a procedure to select for a specific microbial taxon which is known for the particular group of compounds, e.g., isolation of *Streptomycetes* for the production of antibiotics. Otherwise, the isolation procedure may be designed to restrict certain microbial "weeds" and to support the growth of novel types. During the 1980s

significant advances were made in the establishment of taxonomic databases describing the properties of microbial groups and these databases have been used to predict the cultural conditions that would select for the growth of particular taxa. It gives inputs to screen particular taxonomic group with high probability of being productive. Furthermore, whole-genome sequencing of microorganisms (in 2000s) can give an insight into their cultural requirements. The sequence can be used to construct a metabolic map and thus identify nutrients that the organism cannot synthesize, thereby facilitating the design of a defined medium. Further, the advances in pharmacology and molecular biology also enabled the design of screening tests to identify productive strains among the isolated organisms.

2.4 SCREENING METHODS

1. Primary Screening of Microorganisms

Primary screening may be defined as detection and isolation of the desired microorganism based on its qualitative ability to produce the desired product like antibiotic or amino acid or an enzyme etc. In this process desired microorganism is generally isolated from a natural environment like soil, which contains several different species. Sometimes the desired microorganism has to be isolated from a large population of different species of microorganisms.

The following are some of the important primary screening techniques

1. The crowded plate technique
2. Indicator dye technique
3. Enrichment culture technique
4. Auxanographic technique
5. Technique of supplementing volatile and organic substrates.

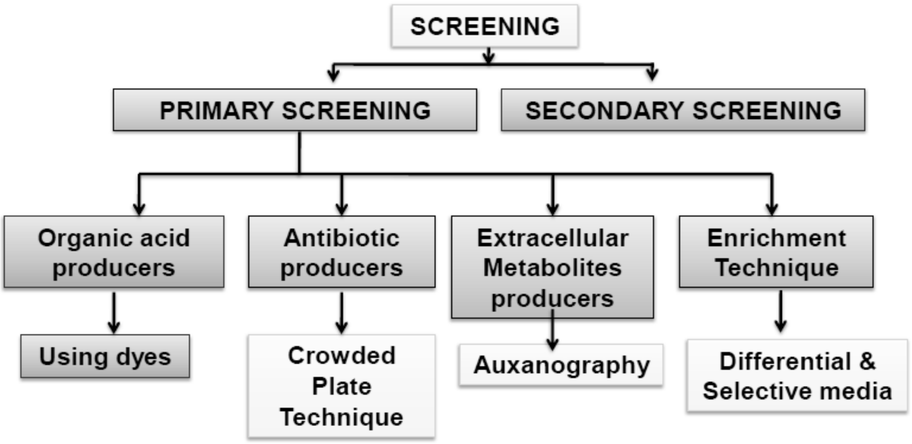


Figure -1: Screening methods.

(i) The Crowded Plate Technique: This technique is primarily employed for detecting those microorganisms, which are capable of producing antibiotics. This technique starts with the selection of a natural substratum like soil or other source consisting of microorganisms. Progressive serial dilution of the source is made. Suitable aliquot of the serial dilution is chosen which is able to produce 300 to 400 individual colonies when plated on an agar plate, after incubation. Such a plate is called as crowded plate.

The antibiotic producing activity of a colony is indicated by no growth of any other bacterial colony in its vicinity. This region of no growth is indicated by the formation of a clear and colorless area around the antibiotic producing microorganism's colony on the agar plate. This region is called as growth inhibitory zone. Such a colony is isolated from the plate and purified either by making repeated sub-culturing or by streaking on a plate containing a suitable medium, before stock culture is made. The purified culture is then tested for its antibiotic spectrum.

However, the crowded plate technique has limited applications, as it will not give indication of antibiotic producing organism against a desired organism. Hence, this technique has been improved later on by employing a test organism to know the specific inhibitory activity of the antibiotic.

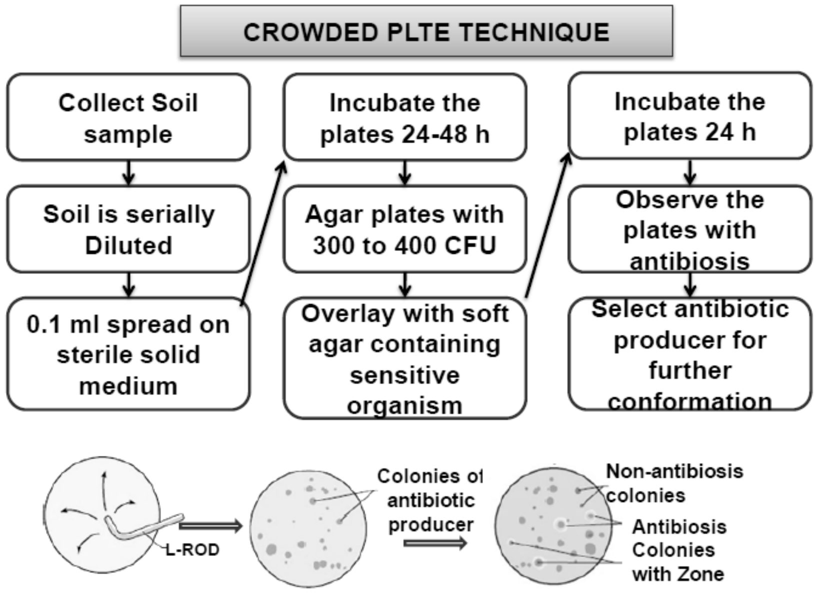


Figure - 2: Crowded Plate technique for antibiotic producer screening.

In this modified procedure, suitable serially diluted soil suspension is spread on the sterilized agar plate to allow the growth of isolated and individual microbial colonies (approximately 30 to 300 per plate) after incubation. Then the plates are flooded with a suspension of test organism and the plates are incubated further to allow the growth of the test organism. The formation of inhibitory zone of growth around certain colonies indicates the antibiotic activity against the test organism. A rough estimation of the relative amounts of antibiotic produced by a microbial colony can be estimated by measuring the diameter of the zone of inhibited test organism's growth. Antibiotic producing colonies are later on isolated from the plate and are purified before putting to further testing to confirm the antibiotic activity of a microorganism.

(ii) Indicator Dye Technique: Microorganisms capable of producing acids or amines from natural sources can be detected using this method by incorporating certain pH indicator dyes such as neutral red or bromothymol blue into nutrient agar medium. The change in the color of a particular dye in the vicinity of a colony will indicate the ability of that colony to produce an organic acid or base.

Production of an organic acid can also be detected by an alternative method. In this method calcium carbonate is incorporated into the agar

medium. The production of organic acid is indicated by the formation of a clear zone around those colonies which release organic acid into the medium. The identified colonies are isolated and purified either by repeated sub-culturing or by streaking methods and a stock culture is made which may be used for further qualitative or quantitative screening tests.

(iii) Enrichment Culture Technique: This technique is generally employed to isolate those microorganisms that are very less in number in a soil sample and possess specific nutrient requirement and are important industrially. This was first designed by Beijerinck to isolate the desired microorganism from a heterogenous microbial population. They can be isolated if the nutrients required by them is incorporated into the medium or by adjusting the incubation conditions. The following are the steps to do the enrichment culture technique,

- Nutrient broth is inoculated with the microbial source material and incubated.
- A small portion of the inoculums is plated on to the solid medium and well isolated colonies are obtained
- Suspected colonies from the plate are subcultured on fresh media and subjected for further testing.
- E.g., Cellulose medium for cellulase producer; Tributyrin medium for lipase producer.

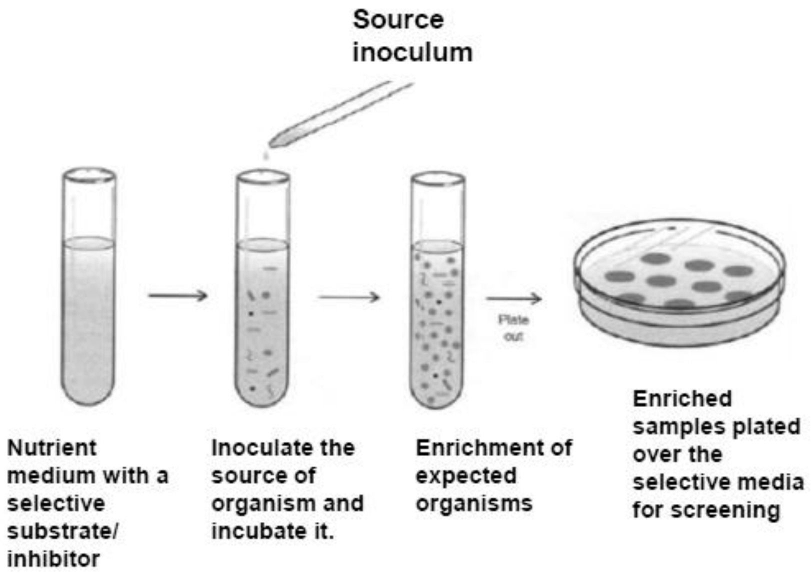


Figure- 3: Enrichment culture technique.

i) **Enrichment liquid culture method:** Enrichment liquid culture is frequently carried out in shake flasks. However, the growth of the

desired type from a mixed inoculum will result in the modification of the medium and therefore changes the selective force which may allow the growth of other organisms, still viable from the initial inoculum, resulting in a succession. The selective force may be reestablished by inoculating the enriched culture into identical fresh medium. This process may be repeated several times before the dominant organism is isolated by spreading a small inoculum of the enriched culture onto solidified medium. The prevalence of an organism in a batch enrichment culture will depend on its maximum specific growth rate compared with the maximum specific growth rates of the other organisms capable of growth in the inoculum. In this technique, fast growing organisms are capable of increasing its cell populations within a short period of incubation. So that the organism with the highest specific growth rate is the desirable one, for it may be suitable to isolate the organism with the restrictive substrate.

The problems of time of transfer and selection on the basis of maximum specific growth rate may be overcome by the use of a continuous process where fresh medium is added to the culture at a constant rate. Under such conditions, the selective force is maintained at a constant level and the dominant organism will be selected on the basis of its affinity for the limiting substrate rather than its maximum growth rate.

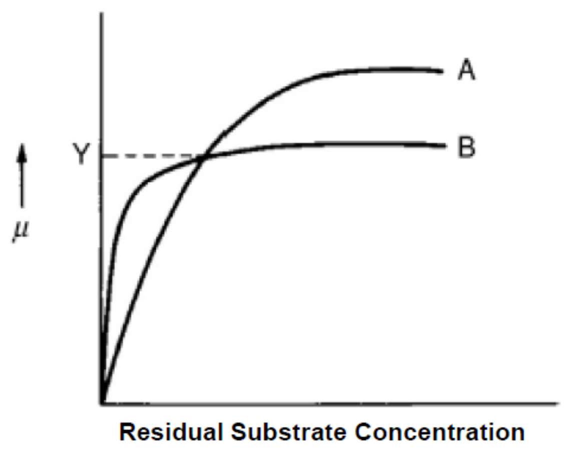


Fig.4: Specific growth of two organisms A and B.

The basic principle of the selection is controlled by the dilution rate and it related to the limiting substrate concentration. For example, in fig.4, a model of the competition between two organisms capable of growth in a continuous enrichment culture is represented. The specific

growth rate is determined by the substrate concentration which is equal to the dilution rate. At dilution rates above Y strain A would be able to maintain a higher growth rate, thus strain A would be selected at dilution rates above Y and strain B would be selected at dilution rates below Y.

This method is suitable for isolating organisms to be used in a continuous-flow commercial process. However, organisms isolated by batch enrichment and purification on solid media normally perform poorly in continuous culture. Hence the enrichment procedure should be designed based on the importance of using the carbon source in the subsequent commercial process as the sole source of organic carbon in the enrichment medium (limited carbon). The addition of vitamins or yeast extract may result in the isolation of strains adapted to using these, rather than the principal carbon source, as energy sources. So the isolation of an organism capable of growth on a simple medium (minimal media with limited carbon source) is a pre request, and should be more resistant to contamination—a major consideration in the design of a commercial continuous process.

The main difficulty in using a continuous-enrichment process is the washout of the inoculum before an adapted culture is established. Alternatively, two-stage chemostat and turbidostat are used for detecting the microbial growth in the continuous enrichment method, where the early washout of the inoculums will be corrected in this method. Example, using continuous enrichment technique yeast lysing enzyme complex producing *Arthrobacter* sp. has been isolated. The technique has been used widely for the isolation of strains capable of degrading environmental pollutants (*Variovax* strains able to degrade trichloroethylene).

ii) **Enrichment cultures using solidified media:** Solidified media have been used for the isolation of certain enzyme producers and these techniques usually involve the use of a selective medium incorporating the substrate of the enzyme that encourages the growth of the producing types. For example, isolation of alkaline proteases producing *Bacillus* from soils, inoculated in various media pHs and to screen the producers correlated with the alkalinity of the soil sample. Colonies that produce a clear zone due to the digestion of the insoluble protein are taken to be alkaline protease producers. The size of the clearing zone could not be used quantitatively to select high producers, as there

was not an absolute correlation between the size of the clearing zone and the production of alkaline protease in submerged culture.

(iv) Auxanotrophic Technique: This technique is employed for the detection and isolation of microorganisms capable of producing certain extracellular substances such as growth stimulating factors like amino acids, vitamins etc. A test organism with a definite growth requirement for the particular metabolite is used in this method. For this purpose, spread a suitable aliquot on the surface of a sterilized agar plate and allow the growth of isolated colonies, after incubation. A suspension of test organism with growth requirement for the particular metabolite is flooded on the above plate containing isolated colonies, which are subjected to further incubation.

The production of the particular metabolite required by the test organism is indicated by its increased growth adjacent to colonies that have produced the required metabolite. Such colonies are isolated, purified and stock cultures are prepared which are used for further screening process.

Steps

i)Preparation of first plate:

- A filter paper strip is put across the bottom of petridish.
- The nutrient agar is prepared and poured on the paper disc and allowed to solidify.
- Soil sample is diluted and proper dilutions are inoculated and incubated.

ii)Preparation of second plate:

- A minimal medium lacking the growth factor is prepared and seeded with the test organism.
- The seeded medium is poured onto a fresh petridish and the plate is allowed to set.
- The agar in the first plate is then carefully lifted with the spatula and placed on the second plate without inverting.
- The growth factors produced by the colonies present on the surface of the first layer of agar can diffuse into the lower layer containing the test organism.
- The zones of stimulated growth of the test organism around the colonies Is an indication that the organism produce growth factor extracellularly.

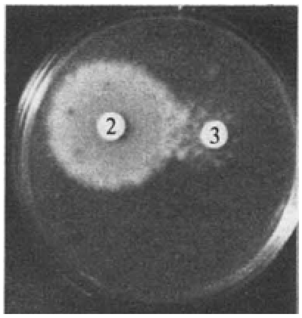


Figure -5. Auxotrophic technique.

(v) Technique of Supplementing Volatile Organic Substances: This technique is employed for the detection and isolation of microorganisms capable of utilizing carbon source from volatile substrates like hydrocarbons, low molecular weight alcohols and similar carbon sources. Suitable dilution of a microbial source like soil suspension are spread on to the surface of sterile agar medium containing all the nutrients except the one mentioned above. The required volatile substrate is applied on to the lid of the petri plates, which are incubated by placing them in an inverted position. Enough vapors from the volatile substrate spread to the surface of agar within the closed atmosphere to provide the required specific nutrient to the microorganism, which grows and form colonies by absorbing the supplemented nutrient. The colonies are isolated, purified and stock cultures are made which may be utilized for further screening tests.

a) Non-Selection of the Desired Characteristic: Microorganisms is isolated and subsequently tested for the desired characteristic in a separate screening process. Waksman (1944) was the first to use extensively for screening antibiotic producing streptomycete and assayed by detecting the inhibition of the growth of pathogenic bacteria as clear zones on an overlay plate, known as the **Waksman platform**, was adopted by pharmaceutical companies throughout the world and gave rise to the "golden age" of antibiotic discovery. However, the efficacy of the method declined as it resulted in the reisolation of strains that had already been screened many times before. Hence, this **"reinvention of the wheel**" syndrome has been minimized in two major ways:

1. Developing procedures to favor the isolation of unusual taxa that are less likely to have been screened previously.
2. Identifying selectable features correlated with the unselectable industrial trait thus enabling an enrichment process to be developed.

Table – 2: Antibacterial Compounds Used in Selective Media for the Isolation of Actinomycetes.

Target Organism	Selective Antibiotic Agent
Micromonospora	Bruneomycin
Microtetraspora	Dihydroxymethylfuratriazone
Micromonospora	Gentamycine
Actinomadura	Kanamycin
Streptomyces	Nitrofurazone
Micromonospora	Novobiocin
Actinoplanes	Tellurite
Micromonospora	Tunicamycin

Table -3: Selective Substrates for the Isolation of Actinomycetes and Antibiotic-Producing Actinomycetes.

Selective Substrates for Actinomycetes	Selective Substrates for Actinomycetes with Antibiotic-Production
Proline	Proline
Glucose (1.0%)	Glucose (1.0%)
Glycerol	Glycerol
Starch	Starch
Humic acid (0.1%)	Humic acid (0.1%)
Propionate (0.1%)	Zinc
Methanol	Alanine
Nitrate	Potassium
Calcium	Vitamins
Cobalt (0.05%)	
Phenol (0.01%)	
Asparagine	

A very wide range of techniques have been developed to increase the probability of isolating novel organisms, including the pretreatment of soil such as drying, heat-treatment, supplementation with substrates, irradiation, and ultrasonic waves. For example, the uses of a collection of bacteriophage are a pretreatment technique to eliminate common

organisms or the detection of environmental bacteriophage as indicators of unusual actinomycetes. In addition antibiotic sensitive pattern of the particular taxa may be used for the selection of the resistant taxa. This knowledge can then be used to optimize the isolation medium and the cycle begins again.

Stepwise discrimination analysis: The statistical stepwise discrimination analysis (SDA) technique is used to design media for the positive selection of antibiotic producing soil isolates. This was achieved by characterizing a collection of eubacterial and actinomycete soil isolates according to 43 physiological and nutritional tests (aminoacid requirements, vitamins and nucleic acids etc). Certain features were identified which, when used as selective factors, enhanced the probability of either isolating actinomycetes or antibiotic-producing actinomycetes (Table-3).

Overproduction Media method: The most desirable isolation medium would be one that selects for the desired types and also allows maximum genetic expression. Cultures grown on such media could then be used directly in a screen. However, it is more common that, once isolated, the organisms are grown on a range of media designed to enhance productivity. The following are the guidelines for "Overproduction Media" design.

a) Prepare a range of media in which different types of nutrients become growth-limiting, for example, C, N, P, O.
b) For each type of nutrient depletion use different forms of the growth-sufficient nutrient.
c) Use a polymeric or complexed form of the growth-limiting nutrient.
d) Avoid the use of readily assimilated forms of carbon (glucose) or nitrogen (NH_4^+) that may cause catabolite repression.
e) Ensure that known cofactors are present (Co^{3+}, Mg^{2+}, Mn^{2+}, Fe^{2+}).
f) Buffer to minimize pH changes.

Genome wide searching: The maximizing gene expression has been a guiding principle in drug discovery; this model could be used for searching novel antibiotic elements in the particular taxa. Example, twenty secondary metabolites genes identified in *Streptomyces coelicolor* genome and the genome sequence of *Streptomyces avermitilis* revealed thirty secondary metabolite gene clusters.

The difficulties of isolating novel organisms and avoiding the "rediscovery" of microbial products were compensated by the development of sophisticated screening methods to make the most of the potential hidden in both microbial broths and synthesized chemical compounds.

2. Secondary Screening of Microorganisms

Primary screening helps in the detection and isolation of microorganisms from the natural substrates that can be used for industrial fermentations for the production of compounds of human utility, but it cannot give the details of production potential or yield of the organism. Such details can be ascertained by further experimentation. The following are the broad range of information pertaining to secondary screening.

a) Ability or potentiality of the organism to produce metabolite that can be used as an industrial organism.
b) The quality of the yield product.
c) The type of fermentation process that is able to perform.
d) Elimination of the organisms, which are not industrially important.

To evaluate the original potential of the isolated microorganisms both qualitative and quantitative analysis are generally conducted. The sensitivity of the test organism towards a newly discovered antibiotic is generally analyzed during qualitative analysis, while the quantum yield of newly discovered antibiotic is estimated by the quantitative analysis.

Evaluation of microbial potentials

Microorganisms isolated in the primary screening are critically evaluated in the secondary screening so that industrially important and viable potentialities can be assessed by the following methods.

1. To determine the product produced by an organism is a new compound or not.
2. A determination should be made about the yield potentialities of various isolated microorganisms that are detected in primary screening for that new compound.
3. It should determine about the various requirements of the microorganism such as pH, aeration, temperature etc.

4. It should detect whether the isolated organism is genetically stable or not.
5. It should reveal whether the isolated organism is able to destroy or alter chemically their own fermentative product by producing adaptive enzymes if they accumulate in higher quantities.
6. It should reveal the suitability of the medium or its constituent chemicals for the growth of a microorganism and its yield potentialities.
7. It should determine the chemical stability of the product.
8. It should reveal the physical properties of the product.
9. It should determine whether the product produced by a microorganism in a fermentative process is toxic or not.
10. Secondary screening should reveal that whether the product produced in fermentation process exists in more than one chemical form. If so, the amount of formation of each chemical formation of these additional products is particularly important since their recovery and sale as byproducts can greatly improve the economic status of the fermentation industry.
11. The new organism should be identified to the species level. This will help in making a comparison of growth pattern, yield potentialities and other requirements of test organism with those already described in the scientific and patent literature, as being able to synthesize products of commercial value.
12. It should select industrially important microorganisms and discard others, which are not useful for fermentation industry.
13. It should determine the economic status of a fermentation process undertaken by employing newly isolated microorganism.

METHODS OF SECONDARY SCREENING

Secondary screening gives very useful information pertaining to the newly isolated microorganisms that can be employed in fermentation processes of commercial value. These screening tests are conducted by using petri dish containing solid media or by using flasks or small fermenters containing liquid media. Each method has some advantages and disadvantages. Sometimes both the methods are employed simultaneously.

Liquid media method is more sensitive than agar plate method because it provides more useful information about the nutritional, physical and production responses of an organism to actual

fermentation production conditions. Erlenmeyer flasks with baffles containing highly nutritive liquid media are used for this method. Flasks are fully aerated with glass baffles and continuously shaken on a mechanical shaker in order to have optimum product yield.

There are several techniques and procedures that can be employed for secondary screening. However, only a specific example of estimation of antibiotic substance produced by species of Streptomyces, is described in the following paragraph. Similar methods could be used for the detection and isolation of microorganisms capable of producing other industrial products.

(i) Giant Colony Technique

This technique is used for isolation and detection of those antibiotics, which diffuse through solid medium. Species of Streptomyces, is capable of producing antibiotics during primary screening. The isolated Streptomyces culture is inoculated into the central area of a sterilized petri plates containing nutrient agar medium and are selected. The plates are incubated until sufficient microbial growth takes place.

Cultures of test organism, whose antibiotic sensitivity is to be measured are streaked from the edges of plate's upto but not touching the growth of Streptomyces and are further incubated to allow the growth of the test organisms. Then the distance over which the growth of different test organisms is inhibited by the antibiotic secreted Streptomyces is measured in millimeters.

The relative inhibition of growth of different test organisms by the antibiotic is called inhibition spectrum. Those organisms whose growth is inhibited to a considerable distance are considered more sensitive to the antibiotic than those organisms, which can grow close to the antibiotic. Such species of Streptomyces, which have potentiality of inhibiting microorganisms is preserved for further testing.

(ii) Filtration Method

This method is employed for testing those antibiotics which are poorly soluble in water or do not diffuse through the solid medium. The Streptomyces is grown in a broth and its mycelium is separated by filtration to get culture filtrate. Various dilutions of antibiotic filtrates are prepared and added to molten agar plating medium and allowed to solidify.

Later on cultures of various test organisms are streaked on parallel lines on the solidified medium and such plates are incubated. The inhibitory effect of antibiotic against the test organisms is measured by their degree of growth in different antibiotic dilutions.

(iii) Liquid Medium Method

This method is generally employed for further screening to determine the exact amount of antibiotic produced by a microorganism like Streptomyces. Erlenmeyer conical flasks containing highly nutritive medium are inoculated with Streptomyces and incubated at room temperature. They are also aerated by shaking continuously and vigorously during incubation period to allow Streptomyces to produce the antibiotic in an optimum quantity.

Samples of culture fluids are periodically withdrawn aseptically for undertaking the following routine checks:

1. To check the suitability of different media for maximum antibiotic production.
2. To determine the value of pH at which there will be maximum growth of the microorganism and antibiotic production.
3. To check for contamination.
4. To determine whether the antibiotic produced is new or not.
5. To check the stability of the antibiotic at various pH levels and temperatures.
6. To determine the solubility of the antibiotic in various organic solvents.
7. To check about the toxicity of the antibiotic against the experimental animals.

After carrying out the above mentioned routine tests further studies are also conducted to know the following additional information:

1. Effect of incubation temperature and antifoaming agents on fermentation.
2. Rate of resistance developed among the test organisms.
3. Checking the antibiotic for its bacteriostatic or bactericidal properties. Its ability to precipitate serum proteins to cause hemolysis of blood or to harm phagocytes.
4. Checking for possibility of inclusion of precursor chemical of the antibiotic production in the medium.
5. Suitability of the organism for mutation and other genetic studies.

2.6 HIGH THROUGHPUT SCREENING METHODS

The Waksman platform were empirical, labor intensive and showed diminishing success rates, as the number of commercially important compounds isolated increased. Thus, new screening methods have been developed that are more precise to identify the targeted desired activity.

Antibiotics are initially detected by growing the potential producer on an agar plate in the presence of an organism (or organisms) against which antimicrobial action is required. Production of the antibiotic is detected by inhibition of the test organism(s). Alternatively, the microbial isolate could be grown in liquid culture and the cell-free broth tested for activity. This approach is extended by using a range of organisms to detect antibiotics with a defined antibacterial spectrum. For example, the kirromycin group of antibiotics discovered from *Bacillus subtilis* and *Streptomyces viridochromogenes* or *Clostridium pasteurianum*. An automated screening method is also available to detect carboxypeptidase inhibitors including novel cephamycin and carbapenem compounds.

Table 4 Enzyme Inhibitors origins from the Actinobacteria

Enzymes	Use of Inhibitors
α-Amylase	Controlling blood glucose and serum insulin levels
β-Glucosidase	Antiviral; control of obesity
Serine proteases	Antifungal
Aspartic proteases	Control of gastroesophageal reflux disease (GERD)
Calpain—a cysteine proteases	Potential in control of neurodegenerative disorders
Angiotensin converting enzyme (ACE)	Antihypertensive
Chitinase	Antifungal
Lipase	Antiobesity
Monoamine oxidase	Antidepressant
Tyrosinase	Skin whitener cosmetic

The increasing frequency of penicillin and cephalosporin resistance among clinical bacteria led to the development of mechanism-based screens for the isolation of more effective antibacterials. Mainly, to

search for a compound that would inhibit β-lactamase and could be incorporated with ampicillin as a combination therapeutic agent. Few enzyme inhibitors are listed in the Table-4.

The progress in molecular biology, genetics, and immunology also contributed extensively to the development of innovative screens in the 1990s, by enabling the construction of specific detector strains, increasing the availability of enzymes and receptors, and constructing extremely sensitive assays. The advances in the screening are parallel to the development of robotic automation systems that facilitated an enormous increase in the rate of screening. Thus, combination of sensitive assays with robotic automation gives rise to what has become known as high throughput screening (HTS). The process is usually carried out using microtiter plates that have been scaled-up from the original 96 well devices to 384, 1536, or 3456 wells. Obviously, the considerable investment in such HTS systems can only be justified if there are sufficient potential agents to test. Agents for testing would include microbial cell-free broths and synthesized chemical compounds. As mentioned previously, the diminishing returns from microbial cultures and a belief that the microbial world had been "over–fished" for pharmaceuticals led the industry to concentrate on compounds synthesized using combinatorial chemistry. Such compounds were available in sufficient numbers to feed the high throughput screens and were deemed a better investment than the isolation of microorganisms and their associated culture and purification of their products.

The whole genome sequencing of the desirable targeted organism will gives the information related to the genes for the growth and metabolic activities, along with the available secondary metabolic pathways involve the biosynthesis of novel metabolites including antibiotics and other industrially valuable compounds. Through this method potential target genes have been identified from the genome sequence analyses. The next step in the process is to clone each target gene into a bacterial host to express their protein that could then be purified and incorporated into a high throughput screen. Most of the proteins are enzymes, the assays of which are available in the literature. Through millions of compound traced around 16 compounds gives positive hits among them only 5 compounds have developed as lead compounds. Leads defined as hits that also have antibacterial activity along with evidence that the mode of action is commensurate with the

inhibition of the target protein, further, the leads undergone the chemical modification to synthesis effective molecules as therapeutics.

Table 5 Examples of High Throughput Screens of Antibacterial Lead.

Target Protein	Function in the Bacterium
β-Keto-acyl carrier protein synthase III (FabH)	Fatty acid synthesis
Enoyl-acyl carrier protein reductase (Fab1)	Fatty acid synthesis
Peptide deformylase	Protein modification
Methionyl-tRNA synthetase	tRNA synthetase
Phenylalanine-tRNA synthetase	tRNA synthetase

A number of reviewers have addressed the difficulties of high-throughput antibiotic screens, they are;

1. The high-throughput antibiotic screens relied predominantly on enzyme assays rather than viable bacteria and one of the major issues in the effectiveness of an antibiotic is its ability to be taken up by the bacterium.

2. This is particularly relevant for Gram negative organisms, which have an outer membrane of negatively charged lipopolysaccharide, thereby blocking access to hydrophobic compounds, and an inner membrane which blocks the uptake of hydrophilic ones.

3. If a compound does pass both barriers, they may be "deported" by multidrug pumps. Thus, the most common reason for the later failure of a "hit" molecule in an in vitro screen is its inability to penetrate the bacterial cell.

Check your Progress-1
Note: Write your answer in the space given below
a. Why do preserve microbes?.
b. How can you maintain microorganisms in your lab?
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2.6 STRAIN IMPROVEMENT METHODS

Natural isolates usually produce very low concentrations of commercially important products. Therefore every attempt is made to

increase the productivity of the chosen organism. Increased yields may be achieved by optimizing the culture medium and growth conditions, but this approach will be limited by the organism's maximum ability to synthesize the product. The potential productivity of the organism is controlled by its genome and, therefore, the genome must be modified to increase the potential yield. Hence, attempts are made to beneficially change the genome of the already improved strain. Thus, the process of strain improvement involves the continual genetic modification of the culture, followed by re-evaluation of its cultural requirements.

Genetic modification may be achieved by either selecting natural variants, or selecting induced mutants or selecting recombinants. There is a small probability of a genetic change occurring each time a cell divides, and then the culture will become more heterogeneous. The heterogeneity of some cultures can present serious problems of yield degeneration because the variants are usually inferior producers compared with the original culture. The variants have been isolated based on superior producers and this has been observed frequently in the early stages in the development of a natural product.

The industrial strains developed by mutation and selection are the starting point for the application of both recombinant DNA technology and genomics to strain improvement and these strains are the basis of many of those used in current processes. These tools for strain improvement have been used for the development of both primary and secondary metabolite fermentations.

METHODS OF IMPROVEMENT OF INDUSTRIAL STRAINS

1) MUTATION

A mutation is a change that occurs in our DNA sequence, either due to mistakes when the DNA is copied or as the result of environmental factors such as UV light and any chemical cause. The process of mutation is called mutagenesis, either spontaneously or induced by any agents to produce mutant strains. Industrially, certain mutants are of higher producers. Industrial mutagenesis has been carried out by mutagenic agents either physical mutagens (ultraviolet, gamma and X-rays) and or chemical mutagens (ethyl methane sulphonate - EMS, nitrosomethyl guanidine – NTG, etc.). For example, UV radiation gives huge percentage of pyrimidine dimers, ionising

radiation causes chromosomal distortion at a very high rate whereas EMS and NTG cause alkylation (Fig.6).

a) Spontaneous mutations

The naturally occurring mutations in the cell are called spontaneous mutations. These mutations occur because of the following reasons (1) mispairing errors (2) depurination (3) deletions and insertion sequences (4) error-prone DNA repair mechanisms. Spontaneous mutations are low frequency mutants usually occur at about 10^{-10} to 10^{-6} per generation per gene. Selection pressure can be used to screen and isolate spontaneous mutants. These mutants can then be further subjected to physical or chemical mutagens to develop an industrial strain. Example, the spontaneous mutation of wild as well as mutant strains of *Penicillium chrysogenum* to improve the production of its metabolite.

b) Induced mutation

This type of mutagenesis involves the usage of physical and chemical mutagenic agents to manipulate the genetic structure of microbes. This is done to improve the desired characteristic of an organism. The procedure of mutagenesis involves following steps: (1) exposure of parent strain to a mutagen (2) random screening of survivors (3) assay of fermentation medium for enhanced formation of product. Every time an improved strain is obtained it is utilised as a parent strain for in next cycle and this process continues until a strain with high throughput is developed. Due to this either deletion or substitution or addition of nucleotide in the newly synthesized DNA, this will change the reading frame of the protein coding genes. Figure -3 describes the types of mutation process. DNA substitution mutations are of two types. Transitions are interchanges of two-ring purines (A= G) or of one-ring pyrimidines (C =T): they therefore involve bases of similar shape. Transversions are interchanges of purine for pyrimidine bases, which therefore involve exchange of one-ring and two-ring structures (Figure -6,7&8).

All the induced mutations are non-target specific. Several mutations may occur in same gene or more than one gene received multiple mutations. However, mutants with higher productive strains should be selected for industrial fermentation. This conventional process is a time consuming one. Hence, alternative methods have been used for industrial strain improvement.

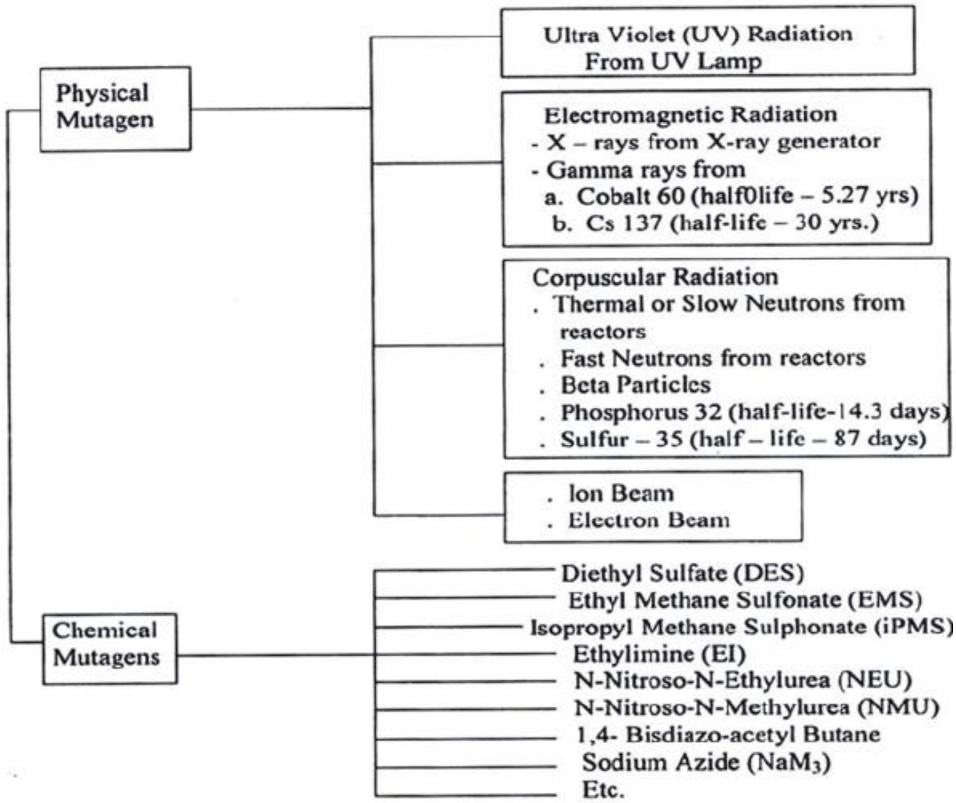


Figure -6: Different types of induced mutagenesis.

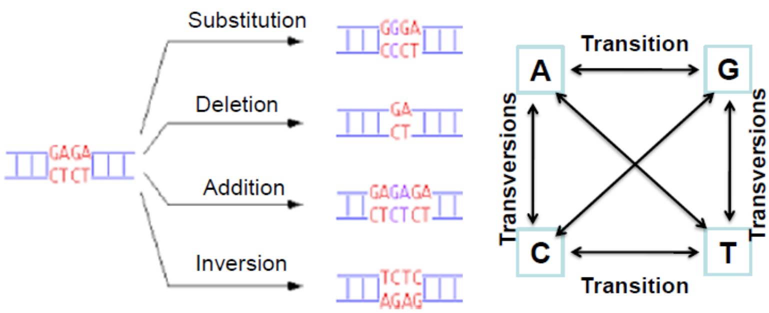


Figure -7: Types of point mutation and Transition and transversion mutation.

c) Directed mutagenesis

The mutations discussed above are totally random and there is no way of knowing the site a certain mutagen is going to affect. In cases, where the gene responsible for the function, that needs improvement, is known we can use site directed mutagenesis. A prerequisite for directed mutagenesis is the knowledge of genes to mutate and the availability of tools for directing the mutagenesis to specific genes controlling the

product formation. It has been reported that actively transcribing genes are more susceptible to mutagenesis, so mutagenesis during the period of highest biosynthetic activity will result in improved product titre mutants. This is carried out optimally in a continuous culture fermenter because the conditions of maximum biosynthetic activity can be judged.

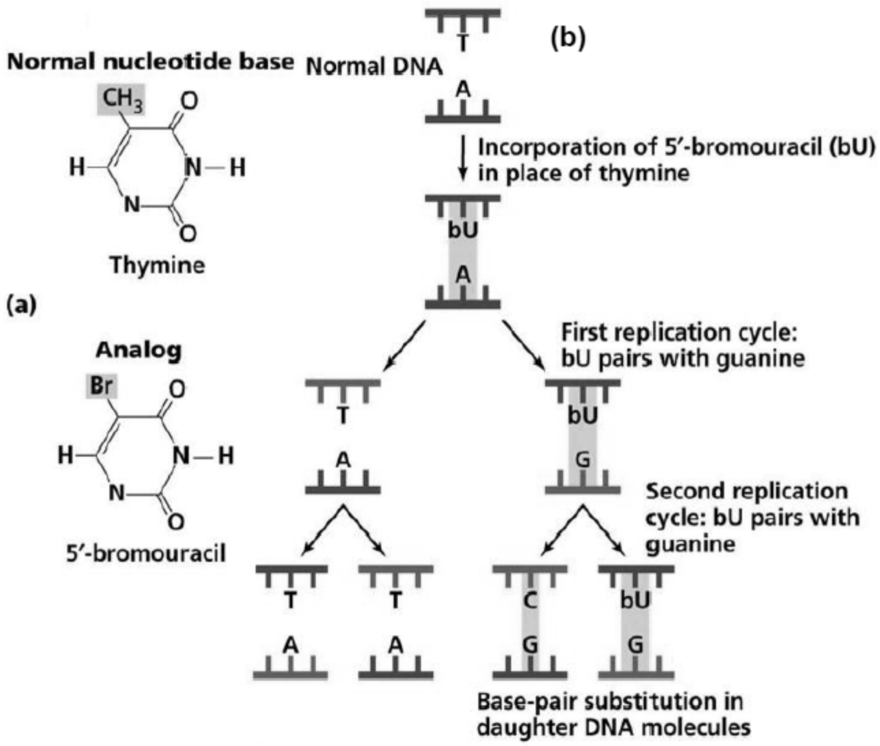


Figure-8: Chemical mutagenesis. Altered base 5'-bromouracil causing substitution mutation.

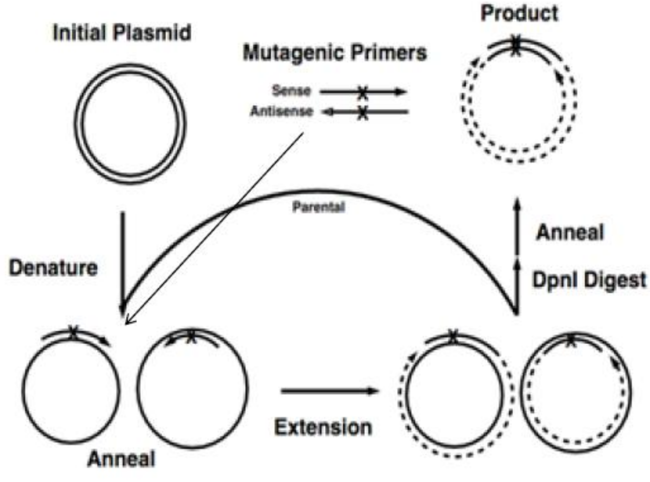


Figure-9: Site directed mutagenesis for targeted genes.

Another approach for directed mutagenesis is the Site-Directed Mutagenesis (SDM)**,** a molecular biology method that is used to make specific and intentional changes to the DNA sequence of a gene (insertions, deletions and substitutions) and any gene products. Also called site-specific mutagenesis or oligonucleotide-directed mutagenesis. The basic procedure requires the synthesis of a short DNA primer. This synthetic primer contains the desired mutation and is complementary to the template DNA around the mutation site so it can hybridize with the DNA in the gene of interest. The mutation may be a single base change (a point mutation), multiple base changes, deletion, or insertion. The single-strand primer is then extended using a DNA polymerase, which copies the rest of the gene. The gene thus copied contains the mutated site, and is then introduced into a host cell as a vector and cloned (Fig. 9). Finally, mutants are selected by DNA sequencing to check that they contain the desired mutation.

RECOMBINATION METHOD

Mutagenesis and recombination are the dominant techniques for strain improvement as compared to recombination. Recombination is more successful if it is used as a complement and not as an alternate technique. Recombination is the process in which two genetically different strains combine to generate a hybrid that is superior and different from either of the parents. Recombination is useful in erasing the neutral and deleterious which arise during random mutagenesis. The major advantage of this technique is that the developed strains are not considered genetically modified microorganisms (GMMO).

Advances in molecular biology techniques have opened up new doors for strain improvement. Recombination DNA technique has initially been used to improve the strains of primary metabolites such as amino acids and extracellular enzymes. Later, it involves cloning of genes and expression of cloned gene into an expression vector to produce specific proteins and enzymes in larger quantity. The expression vector increases the amount of product produced in the presence of an inducer such as: IPTG, etc.

GENETIC ENGINEERING METHOD

Genetic engineering provides an alternative method for improving microbial starter cultures. Through the technology, transfer of a single or cluster of genes in to the desirable host at precised and controllable manner. For the expression, appropriate hosts have been identified,

multifunctional cloning vectors have been constructed, and reliable, high-efficiency gene transfer procedures have been developed (transformation, conjugation or transduction, etc.). Engineered organism such as bacteria, yeast, and molds could also be used for the production of desitable products, including food additives and ingredients, processing aids such as enzymes, and pharmaceuticals. The following are the pre-requisites of this technique.

a) Metabolism and biochemistry of the host: The fundamental understanding of the metabolism and biochemistry of the strain of interest is important to know the metabolic pathways and its regulatory mechanism. This information is essential for the design of genetic improvement strategies, as it provides the rationale for selection of desirable gene(s) and assures that once inserted into a new host, the gene(s) will be appropriately expressed and regulated as predicted.

b) **Transformable hosts:**Plasmid-free, genetically characterized and highly transformable hosts, coupled with multifunctional expression vectors, provide the necessary tools for transfer, maintenance, and optimal expression of cloned DNA in microbial starter cultures. Use of plasmid-free hosts also eliminates plasmid incompatibility problems and the possibility of cointegrate formation between transforming and endogenous plasmids, and should be used as model systems.

c) **Vector systems:** A vector can be defined as a vehicle for transferring DNA from one strain to another. Plasmids are frequently used for this purpose because they are small autonomously replicating circular DNA forms that are stable and relatively easy to isolate, characterize, presence of multiple cloning sites, availability of selectable marker(s) to screen the engineered one. Hence, multifunctional hybrid cloning vectors are suitable for gene manipulation.

d) **Efficient gene transfer systems:** Electroporation, transformation, shotgun method of gene cloning are most preferable. The selectable method must have high transformable efficiency.

e) Expression systems: To optimize expression of cloned genes, efficient promoters, ribosome-binding sites, and terminators must be available at the suitable possiton n the cloning vector. Additionally, signal sequences are essential for secretion of proteins outside the cell may be suitable.

f) Gene of interest: Desirable gene for the expected metabolite should be selected from the native isolates. The complete gene sequences are obtained from the isolates (Example, enzymes, proteins, peptide antibiotic etc.).

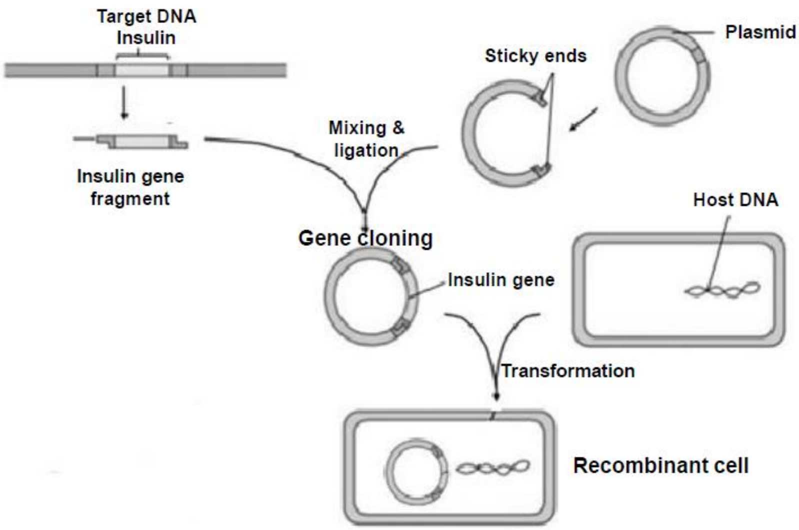


Figure -10: Method of genetic manipulation [human insulin gene cloning].

2.7 SCREENING OF IMPROVED STRAINS

STRAINS PRODUCING PRIMARY METABOLITES

Primary biosynthetic pathways convert the intermediates of carbon catabolic pathways to end products required for the production of biomass. These pathways are regulated by the intermediate metabolites as well as the end product. The improvement in the yield of these products has been achieved predominantly by targeting three sites:

- The control of the terminal pathway
- The supply of precursors from central metabolism
- The supply of NADPH by the recycling of NADP

a) Selection of induced mutants [auxotrophs]

The use of ultraviolet light is only one of a large number of physical or chemical agents that increase the mutation-rate such agents are termed mutagens. The vast majority of induced mutations are deleterious to the yield of the desired product but, a minority is more productive than the parent. The problem of obtaining the high-yielding mutants has been addressed by designing cultural techniques that enable

the separation of the few desirable types from the large number of mediocre producers. Superior productivity is a result of a diversion of precursors into the product and/or a modification of the control mechanisms limiting the level of production. Thus, knowledge of the biosynthetic route and the mechanisms of control of the biosynthesis of the product should enable the prediction of a “blueprint” of the desirable mutant. Based on the above information, the use of selective pressure in mutant isolation is common in the fields of amino acid, nucleotide, and vitamin production. The levels of primary metabolites in bacteria are regulated by feedback control systems such as feedback inhibition, feedback repression, and attenuation.

i. **Feedback inhibition**: The end product of a biochemical pathway inhibits the activity of an enzyme catalyzing one of the reactions (normally the first reaction) of the pathway. Inhibition happened at the allosteric site rather than substrate binding.
ii. **Feedback repression**: The gene level control, the end product of a biochemical pathway acts as a corepressor. The aporepressor is coded by the regulator gene but is only active when combined with the corepressor (the pathway end product). The active complex of co- and aporepressor will bind to the operator site and prevents the transcription of messenger RNA by RNA polymerase, thus repressing the synthesis of an enzyme (or enzymes) catalyzing a reaction (or reactions) of the pathway.
iii. **Attenuation:** It occurs in the control of the biosynthesis of some amino acids and also acts on transcription, but the controlling factor is not the end product (amino acid) but the charged tRNA molecule that delivers the amino acid to the ribosome in the synthesis of a protein. Attenuation controls the synthesis of threonine, isoleucine, valine, leucine, phenylalanine, and histidine, while tryptophan is controlled by both attenuation and repression.

The control of biosynthetic pathways giving rise to a number of end products (branched pathways) is more complex than the control of simple, unbranched sequences. The end products of the same, branched biosynthetic pathway are rarely required by the microorganism to the same extent, so that if an end product exerts control over a part of the pathway common to two, or more, end products then the organism may suffer deprivation of the products not participating in the control. Thus, mechanisms have evolved which enable the level of end products of branched pathways to be controlled without depriving the cell of essential intermediates. The following descriptions of these mechanisms

are based on the effect of the control, which may be arrived at by inhibition, repression, or a combination of both systems.

a) *Concerted or multivalent feedback control:* This control system involves the control of the pathway by more than one end product—the first enzyme of the pathway is inhibited or repressed only when all end products are in excess, as shown in Fig. 11 (a & b).
b) *Cooperative feedback control:* The system is similar to concerted control except that weak control may be affected by each end product independently. Thus, the presence of all end products in excess results in a synergistic repression or inhibition (Fig.11(c).
c) *Cumulative feedback control:* Each of the end products of the pathway inhibits the first enzyme by a certain percentage independently of the other end products. In Fig. 11 (d) both D and F independently reduce the activity of the first enzyme by 50%, resulting in total inhibition when both products are in excess.
d) *Sequential feedback control:* Each end product of the pathway controls the enzyme immediately after the branch point to the product. The intermediates that then build up as a result, control earlier enzymes in the pathway (Fig. 11 (e)). This control is common in the genus *Bacillus sp.*
e) *Isoenzyme control:* Isoenzymes are enzymes that catalyze the same reaction but differ in their control characteristics. Thus, if a critical control reaction of a pathway is catalyzed by more than one isoenzyme, then the different isoenzymes may be controlled by the different end products. Such a control system should be very efficient, provided that control exists immediately after the branch point so that the reduced flow of intermediates is diverted away from the product in excess (fig. 11 (f)).

The ideal industrial microorganism should produce high amount of required growth and product, an understanding is that, the mutant is not restricted to produce the metabolites. Such mutants may be developed in three ways:
1. The organism may be modified such that the end products that control the key enzymes of the pathway are lost from the cell due to some abnormality in the permeability of the cell membrane.
2. The organism may be modified such that it does not produce the end products that control the key enzymes of the pathway.

3. The organism may be modified such that it does not recognize the presence of inhibiting or repressing levels of the normal control metabolites.

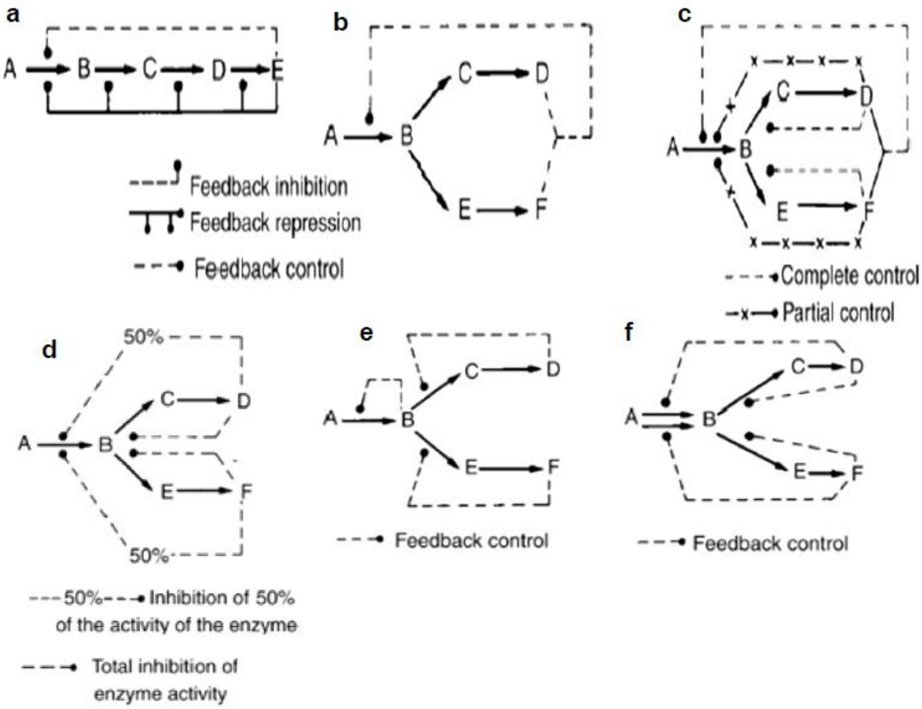


Figure -11: Feedback control or repression of metabolic pathways. The control of biosynthetic pathway converting precursor A to end product E via the intermediates B,C, and D (a); biosynthetic pathway has the concerted effects of products D and F on the first Enzyme on the pathway (b); the control of biosynthetic pathway by the cooperative control by end product D and F (c), a biosynthetic pathway by the cumulative control of products (d), sequencial feed back control (e), and the control of two isoenzymes (catalyze to produce A→B) by end products D and F (f).

One such example is the glutamate fermentation by the mutant *Corynebacterium glutamicum* based on permeability. *C. glutamicum* is a natural biotin auxotroph and provided that the level of biotin in the production medium was below 5 µg dm–3 then the organism would excrete glutamate, but at concentrations of biotin optimum for growth it produced lactate. Biotin is a cofactor of acetyl-CoA carboxylase, a key enzyme in the synthesis of fatty acids. Thus, the effect of biotin limitation was attributed to a partial disruption of the cell membrane's permeability resulting in the passive excretion of glutamate from the cell—the "leak model." Glutamate controls its

own biosynthesis by the feedback repression of phosphoenolpyruvate carboxylase and citrate synthase and both repression and inhibition of NADP-glutamate dehydrogenase with glutamate being maintained at a level of 25–36 μg mg–1 dry weight of cells in the presence of excess biotin. However, under biotin limitation loss of glutamate from the cell prevents its accumulation to inhibiting and repressing levels, resulting in glutamate accumulation in the medium up to a level of 50 g dm^{-3}. Similarly, membrane destabilizers like Tween 80 and penicillin would induce glutamate excretion under excess biotin.

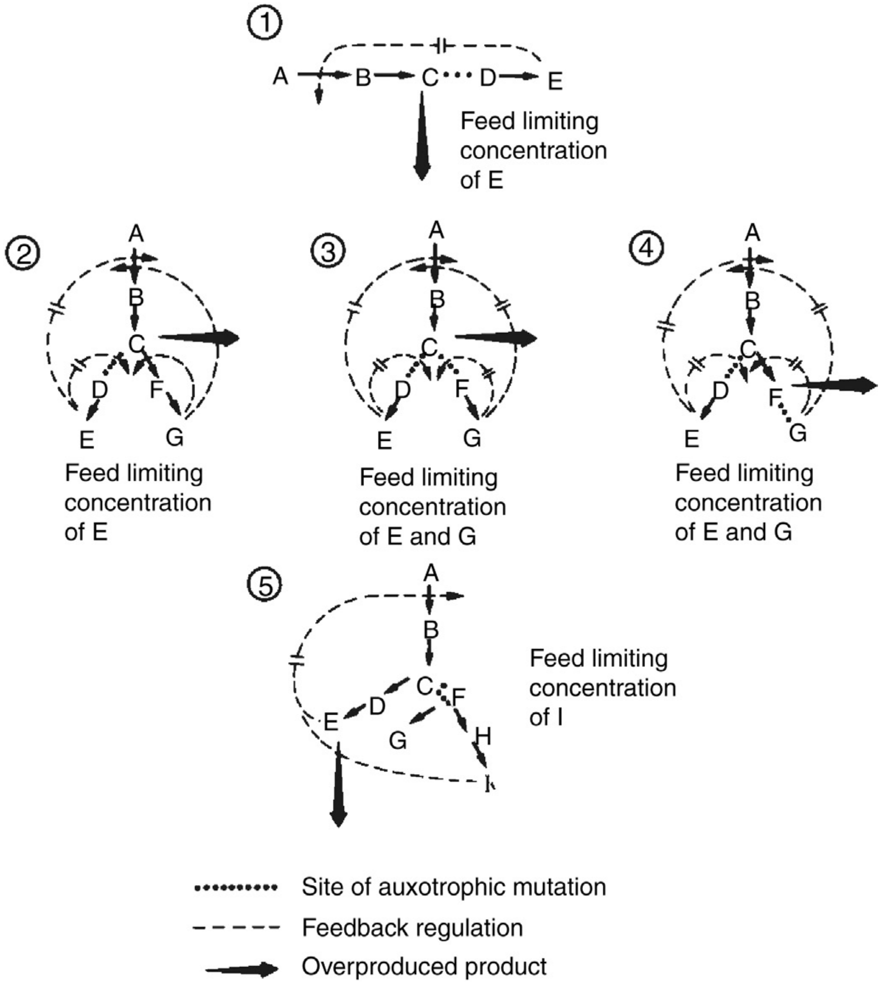


Figure- 12: Overproduction of Primary Metabolites by Decreasing the Concentration of a Repressing or Inhibiting End Product.

ISOLATION OF MUTANTS THAT DO NOT PRODUCE FEEDBACK INHIBITORS OR REPRESSORS

Mutants that do not produce certain feedback inhibitors or repressors have proved useful for the production of intermediates of unbranched pathways; and intermediates and end products of branched

pathways. The figure 12 illustrated the "blue-prints" of several hypothetical mutants do not produce some of the inhibitors or repressors. In the case of Fig. 12(1), the linear pathway is normally controlled by feedback inhibition or repression of the first enzyme of the pathway by the end product, E. However, the organism is auxotrophic for E due to the inability to convert C to D so that control of the pathway is lifted and C will be accumulated provided that E is included in the medium at a level sufficient to maintain growth but insufficient to cause inhibition or repression. Similarly, fig. 12(2) is a branched pathway controlled by the concerted inhibition of the first enzyme in the pathway by the combined effects of E and G. The example shown in Fig. 12(3) is similar to that in Fig. 12(2) except that it is a double auxotroph and requires the feeding of both E and G. Fig. //(4) is, again, the same pathway and illustrates another double mutant with the deletion for the production of G occurring between F and G, resulting in the accumulation of F. Fig. 12(5) illustrates the concerted effect of E and I. The auxotrophic mutant for I and G is inability to convert C to F and, thus, provided G and I are supplied in safe quantities for the growth, the end product, E, will be accumulated.

All the hypothetical examples discussed earlier are auxotrophic mutants and, under certain circumstances, may accumulate relatively high concentrations of intermediates or end products. Therefore, the isolation of auxotrophic mutants may result in the isolation of high-producing strains, provided that the mutation for auxotrophy occurs at the correct site. For example, between C and D in Figs. 12(1) and (2).

Mutant strains are slow growing strains than wild type, hence the antibiotic treatment will reduces the risk of overproducing wild (prototrophs) from the mutants (auxotrophs). Example, penicillin selection of glutamic acid producing *C. glutamicum* . the fact that the ungerminated spores of some organisms are more resistant to certain compounds than are the germinated spores . Thus, by culturing mutated spores in minimal medium only the prototrophs will germinate and subsequent treatment of the spore suspension with a suitable compound would kill the germinated prototrophic spores but leave the ungerminated auxotrophic spores unharmed. The auxotrophic spores may then be isolated by washing, to remove the inhibitor, and cultured on supplemented medium (Fig. 13).

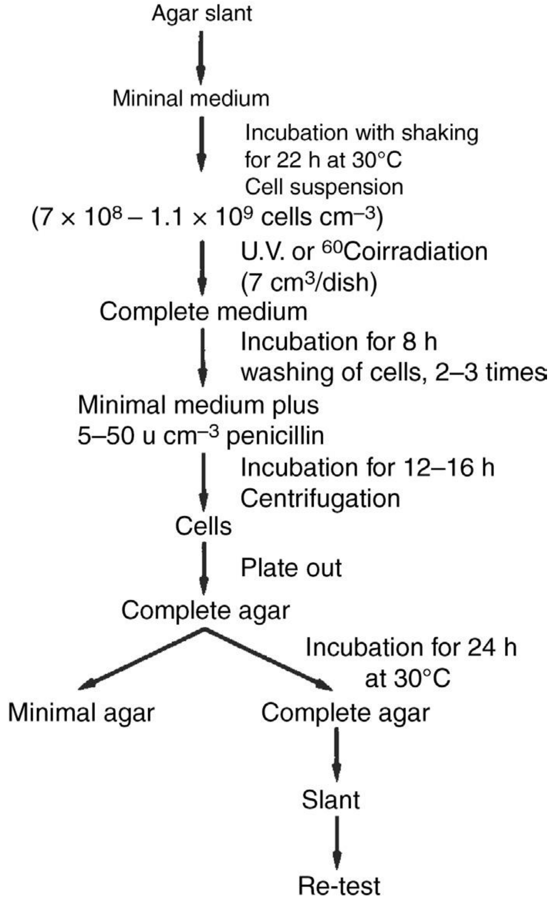


Figure -13. The Use of the Penicillin Selection Method for the Isolation of Auxotrophic Mutants of *C. glutamicum [source: Fermenttation technology, Stanbury]*.

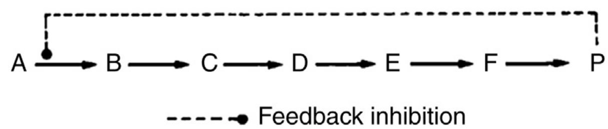


Figure 14: The Control of the Production of an End Product P.

ISOLATION OF MUTANTS THAT DO NOT RECOGNIZE THE PRESENCE OF INHIBITORS AND REPRESSORS

The use of auxotrophic mutants has resulted in the production of many microbial products in large concentrations, but, obviously, such mutants are not suitable for the synthesis of products that control their own synthesis independently. A hypothetical example is shown in Fig. xx where the end product P controls its own biosynthesis by feedback inhibition of the first enzyme in the pathway. If it is required to produce the intermediate F in large concentrations then this may be achieved by the isolation of a mutant auxotrophic for P, blocked between F and P. However, if P is required to be synthesized in large

concentrations it is quite useless to produce an auxotrophic mutant. The solution to this problem is to modify the organism such that the first enzyme in the pathway no longer recognizes the presence of inhibiting levels of P. The isolation of mutants altered in the recognition of control factors has been achieved principally by the use of two techniques:

1. The isolation of analog resistant mutants.
2. The isolation of revertants.

An analog is a compound that is very similar in structure to another compound. Analogs of amino acids and nucleotides are frequently growth inhibitory, and their inhibitory properties may be due to a number of possible mechanisms. For example, the analog may be used in the biosynthesis of macromolecules resulting in the production of defective cellular components. In some circumstances the analog is not incorporated in place of the natural product but interferes with its biosynthesis by mimicking its control properties. For example, consider the pathway illustrated in Fig. 14 where the end product, P, feedback inhibits the first enzyme in the pathway. If P* were an analog of P (which could not substitute for P in biosynthesis) and were to inhibit the first enzyme in a similar way to P, then the biosynthesis of P may be prevented by P* which could result in the inhibition of the growth of the organism.

Mutants may be isolated which are resistant to the inhibitory effects of the analog and, if the site of toxicity of the analog is the mimicking of the control properties of the natural product, such mutants may overproduce the compound to which the analog is analogous. To return to the example of the biosynthesis of P where P* is inhibitory due to its mimicking the control properties of P; a mutant may be isolated which may be capable of growing in the presence of P* due to the fact that the first enzyme in the pathway is no longer susceptible to inhibition by the analog. The modified enzyme of the resistant mutant may not only be resistant to inhibition by the analog but may also be resistant to the control effects of the natural end product, P, resulting in the uninhibited production of P. If the control system were the repression of enzyme synthesis, then the resistant mutant may be modified such that the enzyme synthesis machinery does not recognize the presence of the analog. However, the site of resistance of the mutant may not be due to a modification of the control system; for example, the mutant may be capable of degrading

the analog, in which case the mutant would not be expected to overproduce the end product. Thus, analog resistant mutants may be expected to overproduce the end product to which the analog is analogous provided that:

1. The toxicity of the analog is due to its mimicking the control properties of the natural product.
2. The site of resistance of the resistant mutant is the site of control by the end product.

Resistant mutants may be isolated by exposing the survivors of a mutation treatment to a suitable concentration of the analog in growth medium and purifying any colonies that develop. Sermonti (1969) described a method to determine the suitable concentration. The organism was exposed to a range of concentrations of the toxic analog by inoculating each of a number of agar plates containing increasing levels of the analog with 10^6–10^9 cells. The plates were incubated for several days and examined to determine the lowest concentration of analog which allowed only a very few isolated colonies to grow, or completely inhibited growth. The survivors of a mutation treatment may then be challenged with the predetermined concentration of the analog on solid medium. Colonies that develop in the presence of the analog may be resistant mutants.

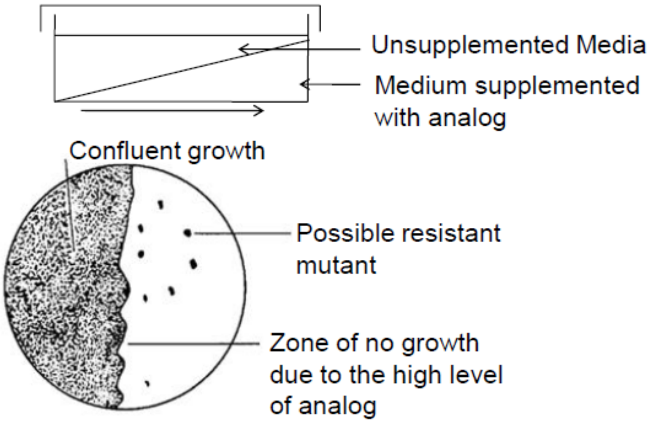


Figure: 15 The Gradient Plate Technique for the Isolation of Analog-Resistant Mutants.

1. The first successful application of genetic engineering techniques to the production of amino acids was obtained in threonine production with *E. coli.*

2. The production of threonine by a threonine analog resistant mutant of *E. coli* K12.
3. Over production was achieved by incorporating a DNA fragment coding for homoserine dehydrogenase from a *B. lactofermentum* threonine producer into a plasmid and introducing the modified plasmid back into the *Brevibacterium.*

2.8 IMPROVEMENT OF STRAINS PRODUCING SECONDARY METABOLITES

Secondary metabolites are the compounds derived from the metabolic intermediates of primary metabolism through a sequential process which is not related to the growth of the producers. Hence, the product would be generated in either slow growing or non-growing populations. For example, polyketides and macrolides are synthesized from acetate moieties, polyisoprenoids from isoprene units, β-lactams from amino acids and a wide range of compounds by nonribosomal peptide synthesis. The control of primary biosynthetic products is dominated by feedback systems that have evolved to ensure that the level of synthesis is commensurate with the requirements of growth. However, the control of secondary metabolism is dominated by systems that switch on biosynthesis (environment).

The most prolific secondary metabolite producers are the Actinobacteria (the actinomycetes), particularly the genus *Streptomyces*. The genes of secondary metabolism tend to be clustered together and many contain only a single regulatory gene that processes the signals that stimulate the transcription of their associated genes. These regulators have been termed "cluster-situated (transcriptional) regulators" or CSRs. It is a protein product that act as transcription factors and switch on the secondary metabolism cluster. For example, the actinorhodin gene cluster is controlled by the protein transcription factor ActII-ORF4 (coded by the gene *actII-ORF4*) and the streptomycin gene cluster by StrR (coded by gene *strR*), both these CSRs being members of the *Streptomyces* Antibiotic Regulatory Protein (SARP) family. The CSRs ultimately respond to an environmental stimulus relayed to it by a signal molecule via a signaling pathway and it is common that several CSRs may be under the control of a pleiotropic regulator (one that causes several responses) that is switched on upstream of the CSRs (fig. 16). The factors that stimulate the onset of secondary metabolism and influence

the CSRs are associated with the shift into substrate limitation, and include the concentration of biomass, starvation, and growth rate.

Further control mechanisms associated with secondary metabolism involve two-component systems (TCSs). A TCS is a signaling system incorporating a sensor and a response regulator. The sensor detects environmental information at the outer surface of the cell membrane and relays information to the response regulator in the cytoplasm that can then initiate a change in gene expression. The detection system is a membrane-bound histidine kinase that phosphorylates itself (using ATP as a phosphate donor) when activated by its environmental stimulus. The phosphoryl group is then transferred to an aspartate residue in the response regulator protein. The activated response regulator may then interact with a specific gene promoter and initiate, or prevent, transcription. The phosphorylated response regulator may be dephosphorylated, thereby returning it to its basal state. The half-life of the phosphorylated response regulator may vary from seconds to hours and thus determines the sensitivity of the control system. The genome of *S. coelicolor* contains 67 two-component systems but the molecular mechanism of action of most is not known. However, it is well known that high phosphate levels can repress secondary metabolism of PhoR-PhoP two-component system in *S. lividans.*

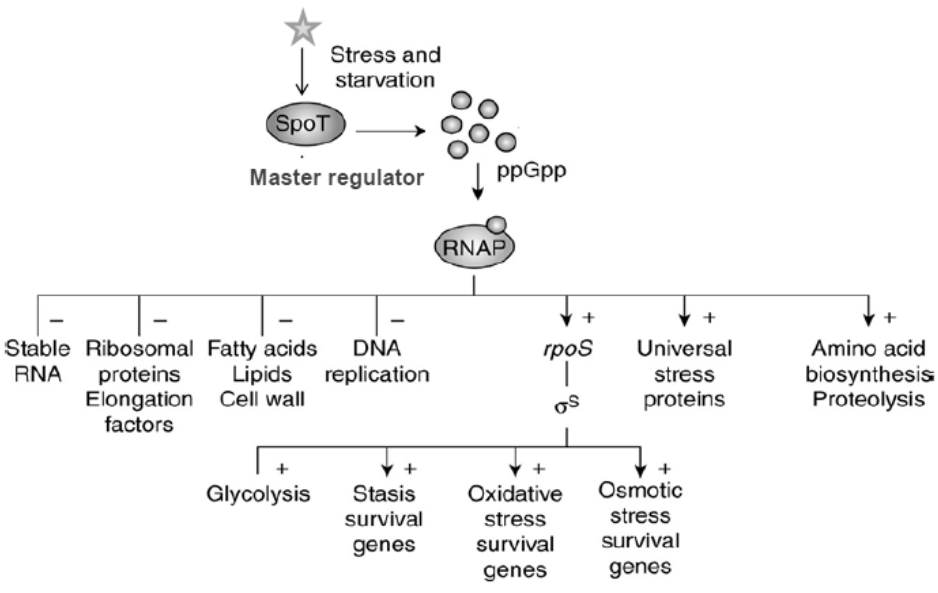


Fig.16: The Stringent Response in *E. coli* in Which ppGpp is Synthesized by stress, starvation and SpoT in Response to Stress or Other Nutrient Depletion

ISOLATION OF MUTANTS PRODUCING IMPROVED YIELDS OF SECONDARY METABOLITES

The empirical approach

The discussion of the directed selection of mutants considered primary products whose biosynthesis and control had been sufficiently understood to prepare "blueprints" of the desirable mutants that then enabled the construction of suitable selection procedures. In contrast, important secondary metabolites were being produced long before their biosynthetic pathways, and certainly the control of those pathways, had been elucidated. Thus, strain improvement programs had to be developed without this fundamental knowledge that meant that they depended on the random selection of the survivors of mutagen exposure. Elander and Vournakis (1986) described these techniques as "hit or miss methods that require brute force, persistence and skill in the art of microbiology." More rational approaches have been developed which reduce the empirical nature of strain-improvement programs. These developments include streamlining the empirical techniques and the use of more directed selection methods. The most common type of shake flask program is, shown in the figure-17.

Directed selection of improved secondary metabolite producers

There are many direct selection methods of improved secondary metabolite producers such as the isolation of auxotrophs, revertants, and analog-resistant mutants. Many secondary metabolites may be considered as end products of branched pathways that also give rise to primary metabolites. Thus, a mutation to auxotrophy for the primary end product may also influence the production of the secondary product. Example, in *P. chrysogenum*, lysine and penicillin share the same common biosynthetic route to α-aminoadipic acid (Fig.18). The lysine (*lys*$^-$) auxotrophs are potentially superior penicillin producers. The explanation of this phenomenon is considered to be the inhibition of homocitrate synthase by lysine resulting in the depletion of α-aminoadipic acid required for penicillin synthesis

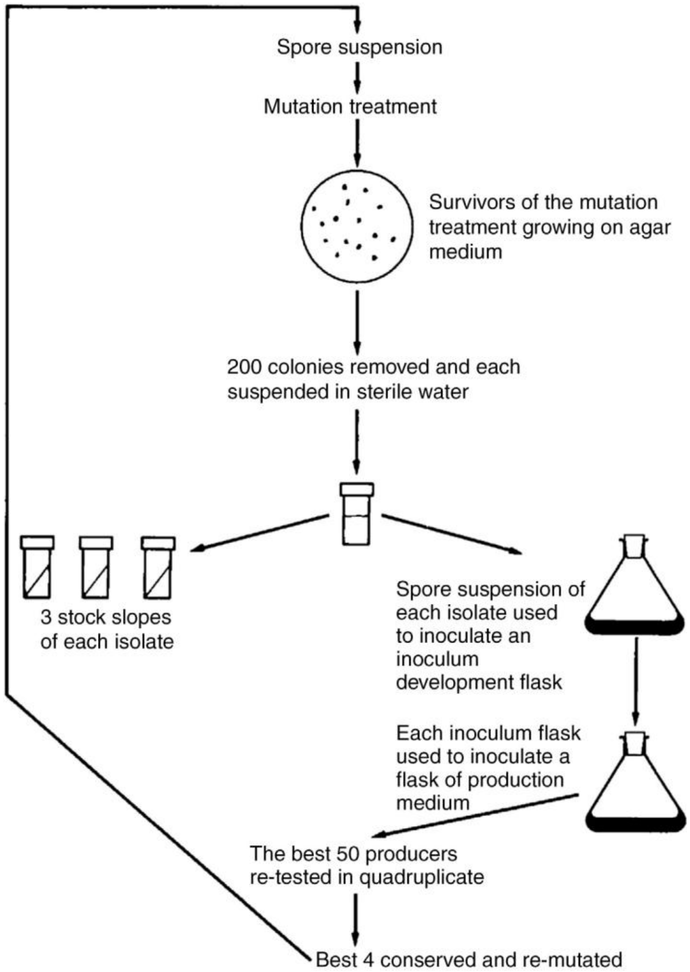


Figure- 17. A Strain-Improvement Program for a Secondary Metabolite Producing Culture.

In the case of analog resistant mutants, a mutant is resistant to the inhibitory effects of an analog, which mimics the control characteristics of the natural metabolite, might overproduce the natural metabolite. This approach has been adopted, or may be adopted, in the field of secondary metabolism. The most obvious application is the enhancement of the production of a precursor of the secondary metabolite. For example, mutants of *Streptomyces lipmanii* resistant to the valine analog (trifluoroleucine) produces higher levels of cephamycin than the parent strain, and appeared to be deregulated for the isoleucine, leucine, valine biosynthetic pathway, indicating that valine may have been a rate-limiting step in cephamycin synthesis.

NOTES

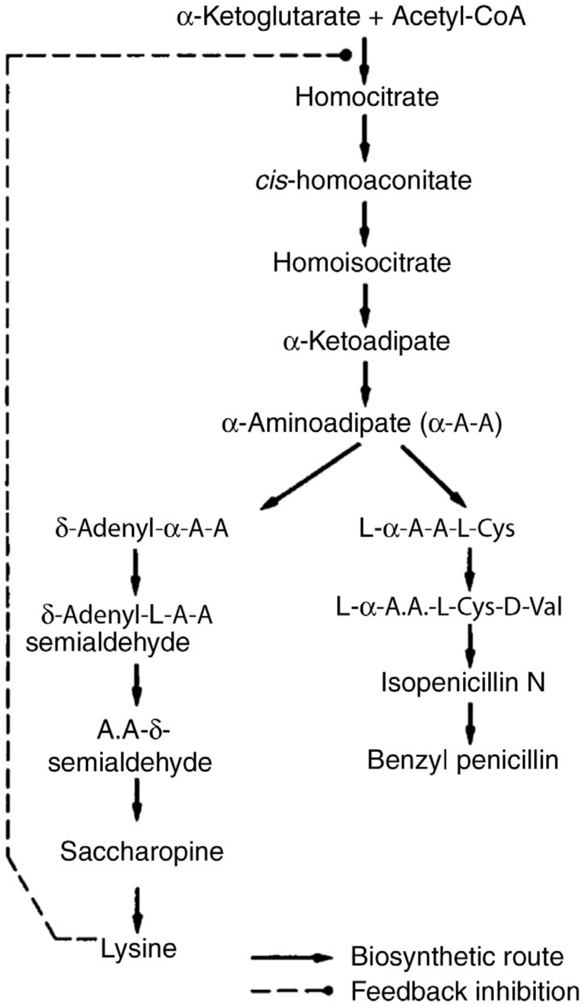


Figure 18: Biosynthesis of Benzyl Penicillin and Lysine in *Penicillium chrysogenum* (Source: Fermentation Technology, Stanbury).

Methionine has been demonstrated to stimulate the biosynthesis of cephalosporin by *Acremonium chrysogenum* and superior producers have been isolated in the form of methionine analog-resistant mutants. Lysine analog resistant mutants have yielded a greater frequency of superior β-lactam antibiotic producers.

Generally, the secondary metabolite itself inhibits its own synthesis. For example, chloramphenicol, aurodox, cycloheximide, staphylomycin, ristomycin, puromycin, fungicidin, candihexin, mycophenolic acid, and penicillin, but its mechanism of inhibition is unknown. Feedback inhibition is also observed in chloramphenicol, which inhibits the first enzyme (arylamine synthetase) involve its biosynthesis.

However, the selection of mutants resistant to feedback inhibition by a secondary metabolite is a difficult process than the isolation of strains resistant to primary metabolic control. It may be achieved by the use of solidified media screening techniques, similar to the miniaturized screening techniques. The technique would involve culturing the survivors of a mutation treatment on solidified medium containing hitherto repressing levels of the antibiotic and detecting improved producers by overlaying the colonies with an indicator organism. The difficulty inherent in this technique is that the incorporated antibiotic, itself, will inhibit the development of the indicator organism. This problem may be overcome by adjusting the depth of the overlay or the concentration of the indicator such that an inhibition zone could be produced only by a level of antibiotic greater than that incorporated in the original medium. Another approach would be to utilize an analog of the antibiotic which mimicked the feedback control by the natural product but which did not have antimicrobial properties.

THE APPLICATION OF THE PARASEXUAL CYCLE FOR STRAIN IMPROVEMENT

Many industrially important fungi do not have a sexual stage and therefore it would appear difficult to achieve recombination in these organisms. However, nuclear fusion and gene segregation could take place outside, or in the absence of, the sexual organs. The process is termed the parasexual cycle (common in the imperfect fungi, example, *A. niger* and *P. chrysogenum)*. In this fungus, nuclear fusion must occur between unlike nuclei in the vegetative hyphae of the organism. Thus, recombination may be achieved only in an organism in which at least two different types of nuclei coexist, that is, a heterokaryon. The heterozygous diploid nucleus resulting from the fusion of the two different haploid nuclei may give rise to a diploid clone and, in rare cases, a diploid nucleus in the clone may undergo an abnormal mitosis resulting in mitotic segregation and the development of recombinant clones which may be either diploid or haploid. The process of recombination during the growth of the heterozygous diploid may occur in two ways: mitotic crossing over, which results in diploid recombinants, and haploidization, which results in haploid recombinants.

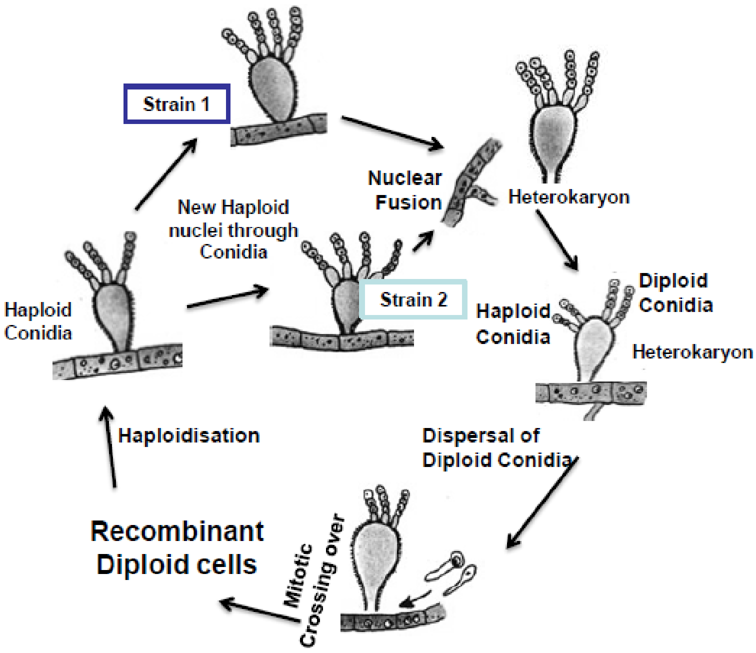


Figure- 19: The parasexual cycle of Fungi imperfecti.

Mitotic crossing over is the result of an abnormal mitosis. The normal mitosis of a heterozygous diploid cell (where $2n = 2$) (Fig. 19). During mitosis, each pair of homologous chromosomes replicate to produce two pairs of chromatids and a chromatid of one pair migrates to a pole of the cell with a chromatid of the other pair. Division of the cell at the equator results in the production of two cells, both of which are heterozygous for all the genes on the chromosome. Therefore, the major components of the parasexual cycle are the establishment of a heterokaryon, vegetative nuclear fusion, and mitotic crossing over or haploidization resulting in the formation of a recombinant. In practice, the occurrence and detection of these stages may be enhanced by the use of auxotrophic markers. The application of the parasexual cycle to industrially important fungi has been hindered by a number of problems.

(1)The influence of the auxotrophic markers (used for the selection of the heterokaryon).

(2)Unpredictable results on the production of some secondary metabolites.

However, the development of protoplast fusion methods in the late 1970s enabled efficient heterokaryon formation to be achieved and removed the major barrier to the application of the parasexual cycle to

strain improvement. The advantages are; the process is more economic, low viscosity, sporulation, and the elimination of unwanted products.

PROTOPLAST FUSION

Fusion of fungal protoplasts appears to be an excellent technique to obtain heterokaryons between strains where conventional techniques have failed, or, indeed, as the method of choice. A demonstration of the use of protoplast fusion for an industrial fungus is the cephalosporin producer, *C. acremonium.* The results of the conventional fusion techniques suggested that nuclear fusion was difficult to achieve. Recombinants would obtain in both sister and divergent crosses. A cross between an asporulating, slow-growing strain with a sporulating fast-growing strain, which produces one-third of the cephalosporin level, however, the recombinant which combined the desirable properties of both strains, that is, a strain which demonstrated good sporulation, a high growth rate, and produced 40% more antibiotic than the higher-yielding parent.

2.9 THE MAINTENANCE OF INDUSTRIALLY IMPORTANT MICROBES

Microbes are required for the production of fermentation products. They are very valuable for specific product. Once industrially valuable microorganism has been isolated and screened they should be grown in pure culture and it becomes necessary to maintain the viability and purity of the microorganism for long duration. Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination. But in industries the mother culture should be maintained for a long duration without any phenotypic and genotypic changes, protect against contamination, and retain viability. There are many culture preservation methods are available and are practice in different industries.

Different techniques are used for maintenance and preservation of different organisms based on their properties. Selected method should also conserve the properties of the organisms.

Techniques for the preservation of microbes broadly divided into two;

1. Methods where organisms are in continuous metabolic active state
2. Methods where organisms are in suspended metabolic state (dehydration and frozen storage)

The main objective of preservation methods is to maintain the viability and genetic stability of the culture by reducing the organism's metabolic rate thereby extending the period between subcultures. Continuous growth includes all techniques that allow the organism to grow and metabolize during storage.

There are several factors influence the viability of cultures during the storage. These include manipulation of growth conditions by limiting carbon, nitrogen and energy sources, lowering the temperature, or preventing dehydration. Further, dehydration or drying can also be used to preserve organisms for long period. It includes,

1. Techniques include air-drying, desiccation in or above a desiccant or drying in a vacuum either from the liquid or frozen state.
2. Frozen storage or cryopreservation is storage at a temperature where the organism is frozen to reduce or completely prevent metabolism and physical change.

Success of the preservation depends on the use of the suitable storage medium, method of cultivation and the age of the culture at the time of preservation.

The method of preservation is mainly of two types:

1. Short- term preservation [serial transfer of organisms to fresh medium, storage at low temperature, maintenance of spores in dry sterile soil etc.], and
2. Long-term preservation [freeze drying or ultrafreezing in liquid nitrogen (-196ºC)].

There is no universal method of preservation that is successful for all microorganisms. The option of preservation method is based on the nature of the microorganism, availability of equipment and skilled personnel and on the preservation purpose. For example the choice may be influenced by the anticipated period of preservation, the number of cultures, frequency of use, maintenance cost and their

application. All preservation methods follow the basic protocol with distinct stages:

1. Culture purity check
2. Preparation of the ampoules (labeling, sterilizing)
3. Growth of the culture
4. Suspension of the cells in preservation medium
5. Dispensing of cell suspension into ampoules
6. Preservation (by method of choice)
7. Ampoule stock storage
8. Update ampoule stock records
9. Ampoule recovery and testing (viability, purity, genetic stability)

A. CONTINUOUS METABOLIC ACTIVE STATE PRESERVATION TECHNIQUE

a) Regular Subculture: Periodic transfer on fresh, sterile media can maintain microbial culture in active state. The culture preserved in this way is maintained by alternate cycles of active growth and storage periods obtained by series of subcultures. Subculture is a common method to all microbiological laboratories; it is known as basic skills of aseptic technique without any special equipment. The frequency of transfer varies with the organism. For example, a culture of *E. coli* needs to be transferred at monthly intervals. After growth for 24 hours at 37ºC, the slants can be stored at low temperature for 20-30 days. To keep the cultures viable, it is necessary to use an appropriate growth medium and a proper storage temperature. The frequency of subculture can be reduced if growing it on a medium containing minimal nutrition lowers the metabolism of the organism. Several factors should be responsible for maintaining a microbial culture by using subculture method. Solid media should be the preference than the liquid as there is a higher chance of contamination in liquid media. Slope cultures rottenly used for preservation but oxygen sensitive bacteria may benefit from stab culture. After subculturing the tubes should be sealed properly. Cotton wool plugged tubes are not adequate, as media will quickly dry out and cultures will be lost.

Regular sub-culturing has many disadvantages, they are:

1. Adequate contamination possibility when large numbers of cultures are involved and the skill of the technical person handle the culture transfer.

2. Change of characteristics- Subculturing can lead to change of characteristics,
3. Mislabeling possibility of cultures may be labeled with the wrong name or number. Labels may become distorted and unrecognizable.
4. Loss of cultures viability – due to the temperature fluctuations in incubation or refrigeration.

b) Paraffin Method (Oil overlay): This is a very simple and cost effective method of preserving cultures of bacteria and fungi for longer time at room temperature. In this method sterile liquid paraffin is poured over the slant culture of microbes and stored upright at room temperature. The layer of paraffin prevents dehydration of the medium and ensures anaerobic conditions. It slows the metabolic activity by reduced growth through reduced oxygen tension. Cultures can also be maintained by covering the agar slants with a layer of sterile mineral oil about half inch above the surface of the slant. The oil must be above the tip of the slanted surface. Mineral oil covered cultures are stored at room temperature or selectively at 0-4ºC. In this method, certain microorganisms have been preserved for more than 10-20 years. The following steps should be consider during this storage,

1. The quality of the oil is very important, as any rancidity or toxic substance is harmful to the organisms.
2. It is preferable to sterilize the oil in the hot air oven at 150ºC to 170ºC for one hour.
3. Unless the oil is well above the uppermost level of the medium, the medium tends to dry out, separate from the wall of the tube and float to the surface of the wall, in which the even the organisms are usually found dead.

c) Storage in sterile soil: Various fungi such as *Fusarium, Penicillium, Alternaria, Rhizopus, Aspergillus* etc. are stored successful in sterile soil. Soil storage involves inoculation of 1.0 ml of spore suspension into soil (that has been autoclaved twice) and incubating at room temperature for 5-10days for growth. This initial growth period allows the fungus to use the available moisture and gradually to become dormant. The culture vessel are then stored at refrigerator. Spraying few soil particles on a suitable medium retrieves the culture.

d) Storage in Silica Gel: Both bacteria and yeast can be stored in silica gel powder at low temperature for a period of 1-2 years. In this method, finely powdered, heat sterilized and cooled silica powder is mixed with a thick suspension of cells and stored at low temperature. The basic principle in this technique is quick desiccation at low temperature, which allows the cell to remain viable for a long period.

e) Storage at refrigerator or cold room storage: Live cultures on a culture medium can be successfully stored in refrigerators or cold rooms, when the temperature is maintained at 4ºC. At this temperature range the metabolic activities of microbes slows down greatly but do not altogether stop. As a result, bacterial metabolism will be very slow and low quantity of nutrients will be utilized. This method cannot be used for a very long time because toxic products get accumulated which can kill the microbes. Refrigerator or cold room storage is of use only for short time preservation of cultures.

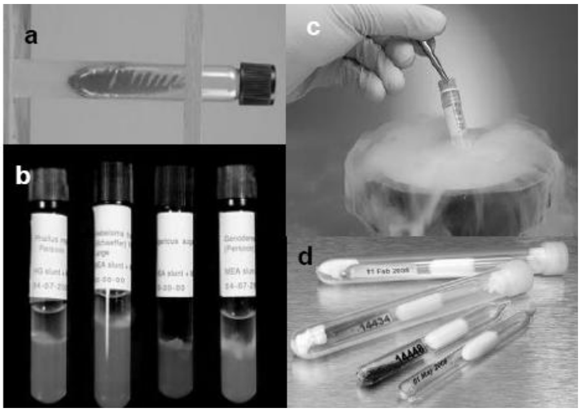


Figure- 20: Various culture maintenance methods. a) Agar slant culture; b) mineral oil storage, c) liquid nitrogen storage, d) Lyophilized vials.

B. METHODS WHERE ORGANISMS ARE IN SUSPENDED METABOLIC STATE

a) Storage by freezing: Freezing is a common process for storage of bacteria. Usually, thick bacterial suspensions can be frozen at a temperature of - 30ºC. The metabolic rates are reduced by lowering the temperature and in the extreme case of storage in liquid nitrogen at -196ºC, are considered to be reduced to nil. Freezing and thawing is a well-known method for actually disrupting cells. Besides, as water is removed during freezing as ice, electrolytes become increasingly concentrated in unfrozen water, and this too may be harmful, since

electrolyte concentrations outside cells become very different from inside those cells, leading to osmotic stress.

Cultures can be preserved very effectively if frozen in the presence of a cryoprotectant, which reduces damage from ice crystals. Glycerol or dimethylsulphoxide (DMSO) are commonly used as cryoprotectants. The simplest way to preserve a culture is to add 15%(v/v) glycerol to the culture and then to store it at -20ºC or -80ºC in a freezer. Cultures can be preserved for a number of years in glycerol, at a temperature of -40ºC in a freezer. In this method, about 2 ml of glycerol solution is added on to the agar slant culture. Shaking can emulsify the culture. Emulsion is then transferred to ampoules, with each ampoule having 5 ml of the culture. These ampoules are placed in a mixture of industrial methylated spirit and carbon dioxide and frozen rapidly to -70ºC. Ampoules are then removed and placed directly in a deep freeze at -40ºC for utilization of the stock cultures. The ampoules are kept in a water bath at 45ºC for about a few seconds and then used for plate cultures. The use of cryogenic storage at ultra-low temperature obtained by freezing in liquid nitrogen at -196ºC has proven to be a simple standardized technique for the preservation of a wide range of microorganisms and mammalian cells.

1. Advantage: Little loss of viability, rapid resuscitation, ready availability as a living suspension and speed of preparation.
2. Disadvantages: The cost of the apparatus and regular supplies of liquid nitrogen, risk of explosion when ampoules are brought into room temperature, loss of large numbers of cultures if careful monitoring of liquid nitrogen levels is not carried out and possible contamination of the liquid nitrogen in the storage container if an ampoule breaks.

b) Storage by freeze drying (Lyophilization): Freeze-drying is the most widely used technique for maintaining bacterial cultures. Freeze drying is also called lyophilization.

When bacterial cultures or virus suspensions are dried and kept in the dry state under suitable conditions. When such materials are dried from liquid state, a high salt concentration is produced in the later stages of drying; this causes denaturation of proteins, death of organisms and deterioration of serum. Freeze-drying or lyophilization whereby the culture or serum is dried rapidly in vacuums from the

frozen state largely avoids the problem. The material is frozen by a suitable method and then dried by sublimation of the ice.

Freeze- drying is a multistage process; it begins with freezing, a temporary stop to metabolic activity, then continues with the removal of water without thawing (sublimation), and ends with a dried product. The dried product is sealed either under vacuum or under an inert gas, can be stored at room temperature with no further metabolic activity until water and nutrients are restored. Freezing must be very rapid, with the temperature lowered to well below 0ºC (e.g., to -20ºC), since slow freezing would prolong exposure to the denaturing influence of the suspending salt solution. The liquid should be frozen in a shallow layer with a large surface available for evaporation. Lyophilized cultures need to be rehydrated according to the manufacturer's directions and maintained viable through frequent transfers or freezing procedures.

Freeze drying involves:

a) Predrying requirements

b) Ampoule preparation (20 minutes under 20lbs pressure)

c) Harvesting the culture (3-5days culture)

d) Primary drying (2-4 hours, more than 90% of free water is removed)

e) Secondary drying (18-20 hours upon which the moisture content is reduced to 1%)

f) Sealing the ampoules (under vacuum sealed with flame)

g) Storage (at 4ºC)

Two types of commercial freeze-dryers are available, (1) the centrifugal and (2) shelf are in common use.

One of the major advantages of freeze-drying is that the ampoules are particularly suitable as a means of distributing cultures because the viability and integrity of the ampoules resist the changes in pressure and temperature in the airmail services.

A disadvantage of freeze-drying is the relatively high capital cost of commercial equipment.

c) Storage by drying methods: Some strains, which are sensitive to freeze-drying, can be preserved by drying from the liquid state rather than the frozen state. A number of methods for drying suspensions of

bacteria for preservation purposes have been developed which are useful in laboratories that cannot afford the expensive equipment used for storing at very low temperatures or for freeze drying, or in which preservation of cultures is performed infrequently.

Some of the following procedures of drying method are mentioned below:

1. Paper Disc: A thick suspension of bacteria is placed on sterile discs of thick absorbent paper, which are then dried over phosphorus pentoxide in a desiccator under vacuum.
2. Gelatin Disc: A thick suspension of bacteria is prepared and added to nutrient gelatin. Drops of the bacterial suspension in gelatin are placed on sterile waxed paper or on a Plastic Petri dish and then dried under vacuum.
3. Pre dried Plugs: Thick suspensions of bacteria are prepared and drops placed on sterile cellophane or on pre dried plugs of peptone, starch or dextran before drying in desiccators.
4. L- Drying: Bacteria in small ampoules are dried from the liquid state using a vacuum pump and desiccant and a water bath to control the temperature. E.g., vesicular arbuscular mycorrhizal fungi. In this method, suspensions of the organisms are dried under vacuum from the liquid state without freezing taking place.

Check your Progress -2

Note: Write your answer in the space given below

c. Define lyophilization.

d. Describe the methods of cooling in microbial preservation

…………………………………………………………………………
…

2.10 QUALITY CONTROL

Quality control is essential in a culture collection and an effective quality control program must be established to ensure viability, purity and genetic stability are achieved in the preservation. Strains selected for use in a biotechnological process usually have a unique set of characters and productive efficiency. Quality control testing is essential to ensure that these characters, which have been

selected or developed, are preserved in as stable and reliable state as possible.

As per the guidelines, cultures of microorganisms should be maintained weekly or monthly transfers to fresh agar slants or stabs, which are kept under refrigeration at 2-8ºC. Transfers must not exceed five passages from the mother culture. In maintaining the challenged organisms, microbiologists must choose the best method for a particular culture to avoid mutations and minimize variability.

2.11 LET US SUM UP

In this unit, you have learnt about the meaning, definition, need, and objectives of industrial microorganisms, their isolation methods, and strain improvement of the organisms, mutant screening, and selective screening of secondary metabolites producers, maintenance of microorganism for long duration. This knowledge would make you understand what is industrial microorganism? How it can be used for microbial fermentations in industries. The concept such as mutation, genetic regulation blue print method, auxotrophy, recombination metods, site directed mutagenesis etc. in strain improvement. The strategies used for maintenance of industrial important microbes have also leant through this chapter. This knowledge might play very important role in your carrier in bioprocess industries.

2.12 UNIT - END EXERCISES

1. List out the methods of strain improvement
2. Explain the methods of screening of industrially important microbes.
3. What are auxotrophs? How auxotrophs are the improved producers of primary metabolites?

2.13 ANSWERS TO CHECK YOUR PROGRESS

a) Microbes are preserved under various conditions including cold preservation, freezing, lyophilization, frequent subculturing, mineral oil method, etc.
b) in our lab microbes are preserved in refrigeration, mineral oil stocks and frequent cubculturing.
c) Lyophilization or freeze drying is a process in which water is removed from a product after it is frozen and placed under a

vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase..

d) Refrigeration and Freezing Refrigerators used in home kitchens or in the laboratory maintain temperatures between 0 °C and 7 °C. This temperature range inhibits *microbial* metabolism, slowing the growth of *microorganisms* significantly and helping *preserve* refrigerated products such as foods or medical supplies.

2.14 SUGGESTED READINGS

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UNIT -III: INDUSTRIAL MEDIA

Structure

3.1 INTRODUCTION

After going through the unit you will be able to;

- Understand the concept of industrial media
- Know the quality of raw materials and the media formulations.
- Enrich the knowledge in media requirements in industrial fermentation, carbon, nitrogen and other growth requirements in mass production of microbes and product.
- Value the role of buffers, antifoam agents in industrial fermentation and optimization of media components.

3.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the concept of fermentation media.
- Know the properties of media components.
- Understand the importance of media, parameters and optimization methods.
- Seed culture media and fermentation media.

3.3 WHY INDUSTRIAL MEDIA?

NOTES

All microorganisms need water, energy sources, sources of carbon and nitrogen, certain mineral elements and perhaps vitamins plus oxygen for its growth, and product fermentation. The use of a good, suitable, and working medium is important to devise a medium for industrial fermentation. Fermentation media is enough in quantity, nutrient rich, will not produce harmful product, without any harm to product recovery and the fermenting organism's full industrial potentials. However, some of the usable industrial media the raw materials not only may support production of the desired product also produce toxic materials, should be avoided. Liquid media are generally employed in industry because they require less space, are more amenable to engineering processes, and eliminate the cost of providing agar and other solid agents.

On large scale fermentation the sources of nutrients may stick on the following criteria:

1. It will produce the maximum yield of product or biomass per gram of substrate used.
2. It will produce the maximum concentration of product or biomass.
3. It will permit the maximum rate of product formation.
4. There will be the minimum yield of undesired products.
5. It will be of a consistent quality and be readily available throughout the year.
6. It will cause minimal problems during media making and sterilization.
7. It will causes minimal problem in other aspects of the production process particularly aeration and agitation, extraction, purification, and waste treatment.

3.4 BASIC REQUIREMENTS OF INDUSTRIAL MEDIA

All microbiological media, whether for laboratory or for industrial purposes must satisfy the needs of the organism in terms of carbon, nitrogen, minerals, growth factors, and water. In addition they must not contain any toxic materials which are inhibitory to the growth of fermenting organism. Suitably the media would be essential to perform a complete analysis of the organism to be grown. The table 1

shows the basic requirements of the major groups of heterotrophic organisms usually employed in industrial fermentation process.

Table-1: Basic growth requirement of microorganisms (% dry weight basis).

Component	Bacteria	Molds	Yeast
Carbon	48 (46-52)	48 (45-55)	48 (46-52)
Nitrogen	12.5 (10-14)	6 (4-7)	7.5 (6-8.5)
Protein	55 (50 –60)	32 (25-40)	40 (35-45)
Carbohydrates	9 (6-15)	49 (40-55)	38 (30-45)
Lipids	7 (5-10)	8 (5-10)	8 (5-10)
Nucleic Acids	23 (15-25)	5 (2-8)	8 (5-10)
Minerals (same for all three organisms)			
Phosphorus		1.0 - 2.5	
Sulfur, magnesium		0.3 - 1.0	
Potassium, sodium		0.1 - 0.5	
Iron		0.01 - 0.1	
Zinc, copper, manganese		0.001 – 0.01	

The first step to consider is an equation based on the stoichiometry for growth and product formation. Thus for an aerobic fermentation:

Carbon and energy source + nitrogen source + O_2 + other requirements → biomass + products + CO_2 + H_2O + heat

This equation should be expressed in quantitative terms, which is important in the economical design of media if component wastage is to be minimal.

Energy sources: Microbes require energy for growth; energy comes from either the oxidation of medium components or from light. Most industrial microorganisms are chemoorganotrophs, therefore the general source of energy will be the carbon substances such as carbohydrates, lipids, and proteins. Some microorganisms can also use hydrocarbons or methanol as their carbon and energy requirements.

Carbon or energy requirements: Carbohydrates are the basic carbon and energy requirements of microorganisms, usually glucose. Several complex carbohydrates such as starch or cellulose may be utilized by some organisms. The energy sources are not being limited to carbohydrates, but may include hydrocarbons, alcohols, or even organic acids. In the composition of the industrial medium the carbon content must be adequate for the production of cells. For most organisms the biomass production is calculated based on the amount of carbohydrate consumed (known as the yield constant) under aerobic conditions, is about 0.5 gm of dry cells per gram of glucose. This means that carbohydrates are at least twice the expected weight of the cells and must be put as glucose or its equivalent compound. The carbon substrate has a dual role in biosynthesis and energy generation.

The carbon requirement for biomass production under aerobic conditions may be estimated from the cellular yield coefficient (*Y*) which is defined as:

QUANTITY OF CELL DRY MATTER PRODUCED / QUANTITY OF CARBON SUBSTRATE UTILIZED

Fast biomass productivity media consists of high concentrations of rapidly metabolized sugars; however, it is often associated with low productivity of secondary metabolites. The major product of a fermentation process will often decide the selection of carbon source, mainly if the product results from the direct dissimilation of the substrate. Some time the simple carbon catabolite affect the biosynthesis of fermented product (catabolic repression), usually by the availability of excess glucose (Table-2).

i)* Carbohydrates*: Generally, carbohydrates as the carbon source in microbial fermentation processes. The most commonly available carbohydrate is glucose, starch, lactose and cellulose. Starch is obtained from maize, cereals, potatoes, and cassava. Hydrolyzed cassava starch is used as a major carbon source for glutamic acid production. The use of starch particles and glucose as an inexpensive medium for ethanol production. Malt extracts may also be prepared from malted grain. Sucrose is obtained from sugar cane and sugar beet. It is commonly used in fermentation media in a very impure form as beet or cane molasses is used for production of ethanol, SCP, organic and amino acids, and some microbial gums. Molasses or sucrose also may be used

for the production of higher value/low-bulk products such as antibiotics, speciality enzymes, vaccines, and fine chemicals.

Table- 2: Catabolite regulation of metabolite biosynthesis (glucose interfering the process)

Metabolite	**Microorganism**
Penicillin	*Penicillium chrysogenum*
Cephalosporin	*Cephalosporium acremonium*
Griseofulvin	*Penicillium griseofulvin*
Aurantin	*Bacillus aurantinus*
Bacitracin	*B. licheniformis*
Puromycin	*Streptomyces alboniger*
Actinomycin	*S. antibioticus*
Neomycin	*S. fradiae*
Cycloserine	*S. graphalus*
Streptomycin	*S. griseus*
α-Amylase	*B. licheniformis*

(a) Corn steep liquor: This is a by-product of starch manufacture from maize. Sulfur dioxide is added to the water in which maize is steeped. At lowered pH inhibits most other organisms, but encourages the development of naturally occurring lactic acid bacteria especially homofermentative thermophilic *Lactobacillus* spp. which raise the temperature to 38-55°C. Under these conditions, much of the protein present in maize is converted to peptides which along with sugars leach out of the maize and provide nourishment for the lactic acid bacteria. Lactic fermentation stops when the SO_2 concentration reaches about 0.04% and the concentration of lactic acid between 1.0 and 1.5%. At this time the pH is about 4. Acid conditions soften the kernels and the resulting maize grains mill better while the gel-forming property of the starch is not hindered. The supernatant drained from the maize steep is corn steep liquor. Before use, the liquor is usually filtered and concentrated by heat to about 50% solid concentration. The heating process kills the bacteria.

Table 3: Chemical Analysis of Corn-Steep Liquor

Total Solids	**51 %w/v**
Acidity as lactic acid	15%w/v
Free reducing sugars	5.6%w/v
Free reducing sugars after hydrolysis	6.8%w/v
Total nitrogen	4%w/v
Amino acids as % of nitrogen	
Alanine	25
Arginine	8
Glutamic acid	8
Leucine	6
Proline	5
Isoleucine	3.5
Threonine	3.5
Valine	3.5
Phenylalanine	2.0
Methionine	1.0
Cystine	1.0
Ash	1.25%w/v
B Vitamins	
Aneurine	41–49 μg g_{-1}
Biotin	0.34–0.38 μg g_{-1}
Calcium pantothenate	14.5–21.5 μg g_{-1}
Folic acid	0.26–0.6 μg g_{-1}
Nicotinamide	30–40 μg g_{-1}
Riboflavine	3.9–4.7 μg g_{-1}

As a nutrient for most industrial organisms corn steep liquor is considered adequate, being rich in carbohydrates, nitrogen, vitamins, and minerals. The composition of a typical sample of corn steep liquor is given in Table 3. As corn steep liquor is highly acidic, it must be neutralized (usually with $CaCO_3$) before use. Corn steep liquor in a low cost media in ethanol production.

(b) Pharmamedia

Also known as proflo, this is a yellow fine powder made from cotton-seed embryo. It is used in the manufacture of tetracycline and some semi-synthetic penicillins. It is rich in protein, (56% w/v) and contains 24% carbohydrate, 5% oil, and 4% ash, the last of which is rich in calcium, iron, chloride, phosphorous, and sulfate.

(c) Distillers solubles

This is a by-product of the distillation of alcohol from fermented grain. It is prepared by filtering away the solids from the material left after distilling fermented cereals (maize or barley) for whiskey or grain alcohol. The filtrate is then concentrated to about one-third solid content to give syrup which is then drum-dried to give distillers soluble. It is rich in nitrogen, minerals, and growth factors (Table 4).

Table 4. Composition of maize distillers soluble

Components	**%**
Moisture	5
Protein	27
Lipid	9
Fibre	5
Carbohydrate	43
Ash (mainly K, Na, Mg, CO3, and P)	11

(d) Molasses

Molasses is a by-product from sugar industries and it is a source of carbohydrate, and is used in many fermentation industries including the production of potable and industrial alcohol, acetone, citric acid, glycerol, and yeasts. There are two types of molasses depending on whether the sugar is produced from the sugar cane (*Saccharum officinarum*) or the beet (*Beta alba*). After the sugar crystallization, along with sugar crystals the thick brown syrup which contains high sucrose is produced, and which is known as 'massecuite'. The massecuite is centrifuged to remove the sugar crystals and the remaining liquid is known as molasses. The first sugar so collected is 'A' and the liquid is 'A' molasses. 'A' molasses is further boiled to extract sugar crystals to yield 'B' sugar and 'B' molasses. This final molasses is known as 'blackstrap molasses'.

The black strap molasses is low-grade and brown in color, and known as raw sugar, cargo sugar, or refining sugar. This raw sugar is further refined, in a separate factory, to remove miscellaneous impurities including the brown color. Cane and beet molasses differ slightly in composition (Table 5). Beet molasses is alkaline while cane molasses is acid.

NOTES

Table 5 Average composition of beet and cane molasses

Materrs	Beet Molasses % (W/W)	Cane Molasses % (W/W)
Water	16.5	20.0
Sugars:	53.0	64.0
Sucrose	51.0	32.0
Fructose	1.0	15.0
Glucose -	14.0	
Raffinose		1.0 -
Non-sugar (nitrogeneous Materials, acids, gums, etc.)	19.0	10.0
Ash	11.5	8.0

Inverted molasses (also known as high test' molasses) is a brown thick syrup liquid used in the distilling industry and containing about 75% total sugars (sucrose and reducing sugars) and about 18% moisture. Strictly speaking, it is not molasses at all but invert sugar, (i.e reducing sugars resulting from sucrose hydrolysis). It is produced by the hydrolysis of the concentrated juice with acid. In the so-called Cuban method, invertase is used for the hydrolysis. Sometimes 'A' sugar may be inverted and mixed with molasses.

(f) Sulfite liquor [waste sulfite liquor, sulfite waste liquor]

Sulfite liquor (also called spent sulfite liquor) is the aqueous effluent resulting from the sulfite process for manufacturing cellulose or pulp from wood. Depending on the type, most woods contain about 50% cellulose, about 25% lignins and about 25% of hemicelluloses. During the sulfite process, hemicelluloses hydrolyze and dissolve to yield the hexose sugars, glucose, mannose, galactose, fructose and the pentose sugars, xylose, and arabinsoe. The acid reagent breaks the chemical bonds between lignin and cellulose; subsequently they dissolve the lignin. Depending on the severity of the treatment some of the cellulose will continue to exist as fibres and can be recovered as pulp. The presence of calcium ions provides a buffer and helps neutralize the strong lignin sulfonic acid. The degradation of cellulose yields glucose. Variable but sometimes large amounts of acetic, formic and glactronic acids are also produced.

Sulfite liquor is used as a medium for the growth of microorganisms after being suitably neutralized with $CaCO_3$ and enriched with ammonium salts or urea, and other nutrients. It has been used for the manufacture of yeasts and alcohol. Some samples do not

contain enough assaimilable carbonaceous materials for some modern fermentations. They are therefore often enriched with malt extract, yeast autolysate, etc.

Nitrogen Sources: Nitrogen is the major elementary material found in proteins including enzymes as well as in nucleic acids hence it is a key element in the cell. Microorganisms mostly use ammonia or other nitrogen salts. The required amount of nitrogen to be added in a fermentation can be calculated from the expected cell mass and the average composition of the microorganisms used. For example, in bacteria the average N content is 12.5%. Therefore to produce 5 gm of bacterial cells per liter would require about 625 mg N (Table 6). Any nitrogen compound which the organism cannot synthesize must be added as media ingredient. In general, inorganic nitrogen salts have been used. Ammonia or the ammonium ion is the preferred nitrogen source for industrial media. In fungal culture, the ammonium ion represses uptake of amino acids by general and specific amino acid permeases. Sometimes, among the mixtures of nitrogen sources, individual nitrogen components may influence metabolic regulation so that there is preferential assimilation of one component until its concentration has diminished. Besides, the use of complex nitrogen sources for antibiotic production has been a common practice (Table-6). At high concentration, it induces the primary metabolism and it favor the trophophase, when it limited, favor the antibiotic production in the idiophase. For the production of polyene antibiotics, soybean meal has been considered a good nitrogen source because of the balance of nutrients, the low phosphorus content, and slow hydrolysis.

Table 6 Best nitrogen sources for some metabolites production

Main Nitrogen Source(s)	Product
Corn-steep liquor	Penicillin
Peanut granules	Bacitracin
Pancreatic digest of gelatine	Riboflavin
Distillers' solubles	Novobiocin
Pharmamedia ,Soybean meal, $(NH_4)_2SO_4$	Rifomycin
Soybean meal, $(NH_4)_2SO_4$Ammonium salt and natural plant nitrogen source	Gibberellins
Dried beef blood or haemoglobin with $(NH_4)_2SO_4$	Butirosin
Soybean meal	Polyenes

(a) Soya bean meal

Soya beans, is an annual legume which is widely cultivated throughout the world in tropical, sub-tropical and temperate regions between 50°N and 40°S. The seeds are heated before being extracted for oil that is used for food, as an antifoam in industrial fermentations. The resulting dried material, soya bean meal, has about 11% nitrogen, and 30% carbohydrate and may be used as animal feed. Its nitrogen is more complex than that found in corn steep liquor and is not readily available to most microorganisms, except actinomycetes. It is used particularly in tetracycline and streptomycin fermentations.

(b) Peanut (groundnut) meal: Various leguminous seeds may be used as a source for the supply of nitrogen in industrial media.

(c) Blood meal: Blood consists of about 82% water, 0.1% carbohydrate, 0.6% fat, 16.4% nitrogen, and 0.7% ash. It is a waste product in abattoirs although it is sometimes used as animal feed.

(d) Fish meal: Fish meal is used for feeding farm animals. It is rich in protein (about 65%) and, minerals (about 21% calcium 8%, and phosphorous 3.5%) and may therefore be used for industrial microbiological media production.

Mineral Requirements: Minerals are very essential for cellular material construction and metabolic activities of all living organisms. The requirements of the mineral nutrients are usually called micronutrients and trace elements. The major mineral elements needed include P, S, Mg and Fe. Trace elements required include manganese, boron, zinc, copper, molybdenum, and Cobalt. Some of the cellular metabolism required the mineral elements as enzyme co-factors. Phosphates are the important mineral nutrient in industrial media. The concentration of phosphate in a medium, particularly laboratory media in shake flasks, is often much higher than that of other mineral components (Table-7). Part of this phosphate is being used as a buffer to minimize pH changes when external control of the pH is not being used. Some secondary metabolic processes have a lower tolerance range to inorganic phosphate than vegetative growth. This phosphate should be sufficiently low as to be assimilated by the end of trophophase. In specific processes, the concentration of certain minerals may be very critical.

Table-7: Range of minerals as salts used for industrial media.

Component	**Range**
KH_2PO_4	1.0–4.0 (part may be as buffer)
$MgSO_4 \cdot 7H_2O$	0.25–3.0
KCl	0.5–12.0
$CaCO_3$	5.0–17.0
$FeSO_4 \cdot 4H_2O$	0.01–0.1
$ZnSO_4 \cdot 8H_2O$	0.1–1.0
$MnSO_4 \cdot H_2O$	0.01–0.1
$CuSO_4 \cdot 5H_2O$	0.003–0.01
$Na2MoO_4 \cdot 2H^2O$	0.01–0.1

Table 8 Trace elements requirements for metabolite production.

Metabolite	**Trace elements**
Gentamicin	Co Mn
Riboflavin	Fe, Co Fe
Mitomycin	Fe
Monensin	Fe
Actinomycin	Fe, Zn
Candicidin	Fe, Zn
Chloramphenicol	Fe, Zn
Neomycin	Fe, Zn
Patulin	Fe, Zn
Streptomycin	Fe, Zn
Citric acid	Fe, Zn, Cu
Penicillin	Fe, Zn, Cu

The inorganic phosphate concentration also influences the production of certain metabolites including bacitracins, citric acid (surface culture), ergot, monomycin, novobiocin, oxytetracycline, polyenes, ristomycin, rifamycin Y, streptomycin, vancomycin, and viomycin. Similarly the concentrations of manganese, iron, and zinc are the most critical in secondary metabolism. Some of the primary and secondary microbial products whose yields are affected by concentrations of trace metals greater than those required for maximum growth (Table-8).

Growth factors: Growth factors include vitamins, amino acids and nucleotides, all the above growth requirements are important for growth and function of cells. Hence the industrial media consist of the above growth requirements at the optimum level. It is important to remember

that if only one vitamin is required it may be occasionally more economical to add the pure vitamin, instead of using a larger bulk of a cheaper multiple vitamin sources. For example, Calcium pantothenate has been used for vinegar production. Some production strains may also require thiamine and biotin as a growth factors.

Chelators (anti precipitant)

Certain media cannot be sterilized by autoclaving due to the formation of a visible precipitate of insoluble metal phosphates. The insoluable precipitates containing all the iron and most of the calcium, manganese, and zinc present in the medium. This problem may be eliminated by incorporating low concentrations of chelating agents such as ethylene diamine tetraacetic acid (EDTA), citric acid, polyphosphates, etc., into the medium. These chelating agents preferentially form complexes with the metal ions in a medium. The metal ions then may be gradually utilized by the microorganism. The fermentation medium added with 25 mg dm^{-3} of EDTA is suitable and it does not cause inhibition of growth of the microorganism which is being cultured.

Water: Water is the major component of almost all fermentation media, and is needed in many of the other supports such as cooling, heating, cleaning, and rinsing. Clean water of consistent composition is therefore required in large quantities from reliable permanent sources. When assessing the suitability of a water supply it is important to consider pH, dissolved salts, and effluent contamination. The mineral content of the water is very important in brewing, and most critical in the mashing process and the types of beer produced. Hard waters with high carbonate content are better for the darker beers such as stouts. Nowadays, the water may be treated by deionization or other techniques and salts added, or the pH adjusted, to favor different beers so that breweries are not so dependent on the local water source.

Under laboratory conditions, it is possible to meet the organism's requirement by the use of purified chemicals since microbial growth is generally limited to a few liters. However, on an industrial scale, the volume of the fermentation could be in the order of thousands of liters. Therefore, pure chemicals are not usually used because of their cost (expenses), unless the cost of the finished material justifies their use. Pure chemicals are however used when industrial media are being developed at the laboratory level. The results of such studies are used in composing the final industrial medium, which is usually made with unpurified raw materials. The unconnected materials present in the

impure raw materials are not used, and it may decrease the quality and quantity of the fermented product, and may indeed be responsible for the final and distinctive property of the product. So, use suitable industrial scale raw material for media formulation.

3.5 STRATEGY FOR THE SELECTION OF RAW MATERIALS FOR INDUSTRIAL MEDIA

The following are the important criteria used to select appropriate substances as raw material for industrial media preparation.

a. Cost of the material: Cheaper raw materials are more competitive for selling price of the final product. The cost of the raw material is directly influencing the cost price of the finished product. For example, lactose is more suitable than glucose in some processes (penicillin production) because of the slow rate of its utilization; it is usually replaced by the cheaper glucose. Due to these economic considerations, the raw materials used in many industrial media are usually waste products from other processes. Corn steep liquor and molasses are the waste products from the starch and sugar industries, respectively.

b. Availability of the raw material at large: The raw material must be readily available in order to produce the product continuously. If it is seasonal or imported, then it must be possible to store it for a reasonable period. Large stocks help to bang the ever rising cost of raw materials; nevertheless large stocks mean that money which could have found use elsewhere is spent in constructing large warehouses or storage depots and in ensuring that the raw materials are not attacked during storage by microorganisms, rodents, insects, etc. There is also the important implication, which is not always easy to realize, that the material being used must be capable of long-term storage without concomitant deterioration in quality.

c. Transportation costs: The closer the source of the raw material to the point of use the more suitable it is for use, if all other conditions are satisfactory.

d. Ease of disposal of wastes resulting from the raw materials: The disposal of industrial waste is rigidly controlled in many countries. Waste materials often find use as raw materials for other industries. Thus, spent grains from breweries can be used as animal feed. But in some cases no further use may be found for the waste from an

industry, directly influencing the chosen raw material. So, select ideal low spent producing substrate for formulation.

e. Uniformity in the quality of the raw material and ease of standardization is important, otherwise the quality of the product and the yield should be varied each industrial process.
f. Adequate chemical composition of medium is important, which helps to monitor the undesirable product formation during the fermentation, otherwise, it reduce the toxins and other co-metabolites production.
g. Presence of relevant precursors in the substrate is important, that will enhance the substrate utilization for the growth and product fermentation. Free aminoacids and simple sugars in the raw material will facilitate the speedy process of trophophase. For example, penicillin G to be produced the medium must contain a phenyl compound (corn steep liquor); cobalt in media for Vitamin B12 production.
h. Satisfaction of growth and production requirements of the microorganisms

Check your Progress-1

Note: Write your answer in the space given below

a. What are the basic requirements of a media?

b. Define energy source.

c. Give any two nitrogen sources.

…………………………………………………………………………
…………………………………………………………………………
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3.6 BUFFERS

The control of pH may be extremely important if optimal productivity is to be achieved. A compound may be added to the medium to serve specifically as a buffer, or may also be used as a nutrient source. Many media are buffered at about pH 7.0 by the incorporation of calcium carbonate (as chalk). If the pH decreases the carbonate is decomposed. Obviously, phosphates which are part of many media also play an important role in buffering. However, high phosphate concentrations are critical in the production of many secondary metabolites (see section: Minerals earlier in this chapter).

The balanced use of the carbon and nitrogen sources will also form a basis for pH control as buffering capacity can be provided by the proteins, peptides, and amino acids, such as in corn-steep liquor. The pH may also be controlled externally by the addition of ammonia or sodium hydroxide and sulfuric acid.

3.7 Other Requirements of Industrial Media

Some components of a fermentation medium help to regulate the production of the product rather than support the growth of the microorganism, they are either called as precursors, inhibitors, and inducers.

PRECURSORS

Precursors are the chemicals, directly incorporated into the desired product when added to the fermentation. For example, a range of different side chains can be incorporated into the penicillin molecule. The addition of corn-steep liquor increased the yield of penicillin from 20 to 100 units cm^{-3}. Corn-steep liquor contains phenylethylamine, which is preferentially incorporated into the penicillin molecule to yield benzyl penicillin (Penicillin G). Addition of phenylacetic acid increases the product yield. Some of the important precursors and the end product is given in the table -9.

INHIBITORS

Inhibitors are the chemical compounds added to fermentations, more of a specific product may be produced, or a metabolic intermediate which is normally metabolized is accumulated. One of the earliest examples is the microbial production of glycerol. Glycerol production depends on modifying the ethanol fermentation by removing acetaldehyde. The addition of sodium bisulfite to the broth leads to the formation of the acetaldehyde bisulfite addition compound (sodium hydroxy ethyl sulfite). Since acetaldehyde is no longer available for reoxidation of NADH2, its place as hydrogen acceptor is taken by dihydroacetone phosphate, produced during glycolysis. The product of this reaction is glycerol-3-phosphate, which is converted to glycerol. Few inhibitors and its applications in industrial fermentation is given in Table-10.

Table-9: Precursors for Industrial fermentation

Precursor use	**Microorganism**	**Product**
Phenylacetic-acid	*Penicillium chrysogenum*	Penicillin G
Phenoxy acetic acid	*Penicillium chrysogenum*	Penicillin V
Chloride	*Streptomyces aureofaciens*	Chlortetracycline
Chloride	*Penicillium griseofulvin*	Griseofulvin
Propionate	*Lactobacillus bulgaricus*	Riboflavin
Cyanides	*Streptomyces* spp.	Vitamin B12
β-Tononones	*Phycomyces blakesleeanus*	Carotenoids
α-Amino butyric acid	*Bacillus subtilis*	l-Isoleucine
Anthranilic acid	*Hansenula anomala*	l-Tryptophan
Nucleosides and bases	*Streptomyces tendae*	Nikkomycins
Dihydronovobionic acid	*Streptomyces* sp.	Dihydronovo-biocin
dl-α-Amino butyric acid	*Tolypocladium inflatum*	Cyclosporin A

Table 10 Specific and general inhibitors used in fermentations

Inhibitor	**Main Effect**	**Product**	**Microorganism**
Sodium bisulfite	Acetaldehyde production repressed	Glycerol	*Saccharomyces cenutsiae*
Bromide	Chlortetracycline formation repressed	Tetracycline	*Streptomyces aureofaciens*
Penicillin	Cell wall permeability	Glutamic acid	*Micrococcus glutamicus*
Alkali metal/ pH below 2.0	Oxalic acid repressed	Citric acid	*Aspergillus niger*
Various inhibitors	Various effects with different inhibitors	Valine	*Breuibacterium roseum*
Diethyl barbiturate	Other rifamycins inhibited	Rifamycin B	*Nocardia mediterranei*
Ethionine	Affects one-carbon transfer reactions	7-Chloro-6 de-methyltetracycline	*Streptomyces aureofaciens*

INDUCERS

Majority of the industrial enzymes are indusable enzymes. Enzyme production may be in the presence of specific substrates. Inducers are often substrates such as starch or dextrins for amylases, maltose for pullulanase, and pectin for pectinases (Table-11). Some inducers are very potent, such as isovaleronitrile inducing nitralase. Substrate analogs that are not attacked by the enzyme may also serve as enzyme inducers. For example, maltodextrins will induce amylase and fatty acids induce lipase. However, the cost may prohibit their use as inducers in a commercial process.

Table 11 Some examples of industrially important enzyme inducers

Inducer	Microorganism	Enzyme
Starch	*Aspergillus* spp.	α-Amylase
Maltose	*Bacillus subtilis*	
Maltose	*Aerobacler aerogenes*	Pullulanase
Yeast mannans	*Streptomyces griseus*	α-Mannosidase
Phenylacetic acid	*Escherichia coli*	Penicillin acylase
Various proteins	*Streptococcus* spp.	Proteases
	Streptomyces spp.	
	Asperigillus spp.	
	Mucor spp.	
Cellulose	*Trichoderma viride*	Cellulase
Pectin	*Aspergillus* spp.	Pectinases
Isovaleronitrile	*Rhodococcus rhodochrous*	Nitralase

One commercial system that has been developed is based on the *alc* A promoter in *Aspergillus nidulans* to express human interferon $\alpha 2$. This can be induced by volatile chemicals, such as ethylmethyl ketone, which are added when biomass has increase to an adequate level and the growth medium contains a non-repressing carbon source or low non-repressing levels of glucose. Methylotrophic yeasts such as *Hansenula polymorpha* and *Pichia pastoris* may be use methanol inducer for biomass production.

3.8 ANTIFOAM COMPOUNDS

In most microbiological processes, foaming is a problem. It may be due to a component in the medium or some factors or compounds produced by the microorganism. The most common cause of foaming is the presence of proteins in the medium, such as corn-steep liquor, Pharmamedia, yeast extract, meat extract, peanut meal or soybean meal. These proteins may denature at the air–broth interface and form a skin which does not rupture readily. The foaming can cause removal of cells from the medium and autolysis. If abandoned, it causes severe physical and biological problems, including, reduction in the working volume, low oxygen tension, improper nutrient distribution and low heat transfer rates, which leads to loss of product. The patterns of foaming in fermentations:

1. Foaming remains at a constant level throughout the fermentation. Initially it is due to the medium and later due to microbial activity.

2. The foaming falls slightly in the early stages of the fermentation then rises. There are very slight effects caused by the medium but the major effects are due to microbial activity.
3. The fermentation has a low initial foaming capacity which rises. These effects are due solely to microbial activity.
4. A more complex foaming pattern during the fermentation which may be a combination of two or more of the previously described patterns.

If excessive foaming is removed by:

1. Using a defined medium and a modification of some of the physical parameters (pH, temperature, aeration, and agitation). This assumes that the foam is due to a component in the medium and not a metabolite.
2. The foam is unavoidable and antifoam should be used. This is the more standard approach.
3. To use a mechanical foam breaker.

Antifoams are surface active agents, reducing the surface tension in the foams and destabilizing protein films by: (1) hydrophobic bridges between two surfaces, (2) displacement of the absorbed protein, and (3) rapid spreading on the surface of the film.

Ideal antifoam should have the following properties:

1. Should disperse readily and have fast action on an existing foam.
2. Should be active at low concentrations.
3. Should be long acting in preventing new foam formation.
4. Should not be metabolized by the microorganism.
5. Should be nontoxic to the microorganism.
6. Should be nontoxic to humans and animals.
7. Should not cause any problems in the extraction and purification of the product.
8. Should not cause any handling hazards.
9. Should have no effect on oxygen transfer.
10. Should be heat sterilizable.

The following compounds are act as antifoam agents:

1. Alcohols; stearyl and octyl decanol.
2. Esters, Silicones, Sulfonates.
3. Fatty acids and derivatives, including cottonseed oil, linseed oil, soy-bean oil, olive oil, castor oil, sunflower oil, rapeseed oil, and cod liver oil.

4. Miscellaneous; alkaterge C, oxazaline, polypropylene glycol.

These antifoams are generally added when foaming occurs during the fermentation. Because many antifoams are of low solubility they need a carrier such as lard oil, liquid paraffin, or castor oil, which may be metabolized and affect the fermentation process.

3.9 OPTIMIZATION OF THE FERMENTATION MEDIA

Optimization is an important event in industrial fermentation. It could be considered that, all the physical and chemical (nutritional) parameters positively support the biomass production and the highest yield of desirable product. There are two different approaches used for optimization of fermentation medium, 1) classical one factor one time approach, and 2) multifactor single time statistical optimization method.

Medium optimization by the classical method of changing one independent variable (nutrient, antifoam, pH, temperature, etc.) while fixing all the others at a certain level can be extremely time consuming and expensive for a large number of variables. To make a full factorial search which would examine each possible combination of independent variable at appropriate levels could require a large number of experiments, *x'*, where *x* is the number of levels and *n* is the number of variables. This may be quite appropriate for three nutrients at two concentrations (23 trials) but not for six nutrients at three concentrations. In this instance 36 (729) trials would be needed. Industrially the aim is to perform the minimum number of experiments to determine optimal conditions. Other alternative strategies must therefore be considered which allow more than one variable to be changed at a time, called factorial design.

When more than five independent variables are to be investigated, the Plackett– Burman design (1946) may be used to find the most important variables in a system, which is then optimized in further studies. This technique allows for the evaluation of $X - 1$ variables by X experiments. X must be a multiple of 4, for example, 8, 12, 16, 20, 24, etc. Usually one determines how many experimental variables need to be included in an investigation and then selects the Plackett–Burman design. Any factors not assigned to a variable can be designated as a dummy variable. Alternatively, factors known to not

have any effect may be included and designated as dummy variables (Table-12).

Table 12 Plackett–Burman Design for Seven Variables

Run	Variables							Yield
	A	B	C	D	E	F	G	
1	H	H	H	L	H	L	H	1.1
2	L	H	H	H	L	H	L	6.3
3	L	L	H	H	H	L	H	1.2
4	H	L	L	H	H	H	L	0.8
5	L	H	L	L	H	H	H	6.0
6	H	L	H	L	L	H	H	0.9
7	H	H	L	H	L	L	H	1.1
8	L	L	L	L	L	L	L	1.4

(Source: Fermentation Technology, Stanbury)

Table 12 shows a Plackett–Burman design for seven variables (*A*–*G*) at high and low levels in which two factors, *E* and *G,* are designated as “dummy” variables. These can then be used in the design to obtain an estimate of error. Normally three dummy variables will provide an adequate estimate of the error. Each horizontal row represents a trial and each vertical column represents the H (high) and L (low) values of one variable in all the trials. This design requires that the frequency of each level of a variable in a given column should be equal and that in each test (horizontal row), the number of high and low variables should be equal. Consider the variable *A;* for the trials in which *A* is high, *B* is high in two of the trials and low in the other two. Similarly, *C* will be high in two trials and low in two, as will be for all the remaining variables. For those trials in which *A* is low, *B* will be high two times and low two times. This will also apply to all the other variables. Thus, the effects of changing the other variables cancel out when determining the effect of *A*. The same logic then applies to each variable. However, no changes are made to the high and low values for the *E* and *G* columns. The trials are carried out in a randomized sequence.

Table 13 Analysis of the Yields Shown in Table 4.16 (Nelson, 1982)

Factor	***A***	***B***	***C***	***D***	***E***	***F***	***G***
O(H)	3.9	14.5	9.5	9.4	9.1	14.0	9.2
O (L)	14.9	4.3	9.3	9.4	9.7	4.8	9.6
Difference	–11.0	10.2	0.2	0.0 –	0.6	9.2	–0.4
Effect	–2.75	2.55	0.05	0.00 –	0.15	2.30	–0.10
Mean square	15.125	13.005	0.005	0.00	0.045	10.58	0.02

Mean square for error = 0.045 + 0.020 / 2 = 0.0325

This procedure will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in a more detailed study to determine the optimum values to use.

The stages in analyzing the data (Tables 10 and 11) using the above example are as follows,

1. Determine the difference between the average of the *H* (high) and *L* (low) responses for each independent and dummy variable.

Therefore the difference = $\Sigma A\,(H) - \Sigma A\,(L)$.

The effect of an independent variable on the response is the difference between the average response for the four experiments at the high level and the average value for four experiments at the low level.

Thus the effect of =

$$A = \frac{\Sigma A\,(H)}{4} - \frac{\Sigma A\,(L)}{4}$$
$$= \frac{2(\Sigma A\,(H) - \Sigma A\,(L))}{8}$$

This value should be near zero for the dummy variables.

2. Estimate the mean square of each variable (the variance of effect).

For *A* the mean square will be = $\dfrac{(\Sigma A(H) - \Sigma A(L))^2}{8}$

3. The experimental error can be calculated by averaging the mean squares of the dummy effects of *E* and *G*.

Thus, the mean square for error = 0.045 + 0.020 /2 = 0.0325

This experimental error is not significant.

4. The final stage is to identify the factors which are showing large effects. In the example, this was done using an *F*-test for

Factor mean square/ Error mean square

This gives the following values:

$$A = \frac{15.125}{0.0325} = 465.4,$$
$$B = \frac{13.005}{0.0325} = 400.2,$$
$$C = \frac{0.0500}{0.0325} = 1.538,$$
$$D = \frac{0.0000}{0.0325} = 0.00,$$
$$F = \frac{10.580}{0.0325} = 325.6.$$

.

Though, *A*, *B*, and *F* have been identified as the most important factors. The next stage would then be the optimization of the concentration of each factor, which will be discussed later.

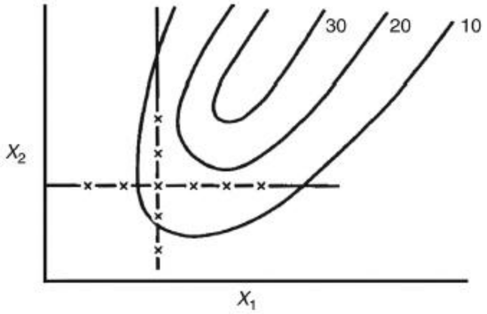


Figure-1: Response surface by one factor at a time.

The next stage in medium optimization would be to determine the optimum level of each key independent variable which has been identified by the Plackett–Burman design. This may be done using response surface optimization techniques. Response surfaces are similar to contour plots or topographical maps. While topographical maps show lines of constant elevation, contour plots show lines of constant value. Thus, the contours of a response surface optimization plot show lines of identical response. In this context, response means the result of an experiment carried out at particular values of the variables being investigated.

The axes of the contour plot are the experimental variables and the area within the axes is termed the response surface. To construct a contour plot, the results (responses) of a series of experiments employing different combinations of the variables are inserted on the surface of the plot at the points delineated by the experimental conditions. Points giving the same results (equal responses) are then joined together to make a contour line. In its simplest form two variables are examined and the plot is two dimensional. It is important

to appreciate that both variables are changed in the experimental series, rather than one being maintained constant, to ensure that the data are distributed over the response surface. In Fig. 1, the profile generated by fixing *X*l and changing *X*2 and then using the best *X*2 value and changing *X*1 constitutes a cross, which may not encroach upon the area in which the optimum resides.

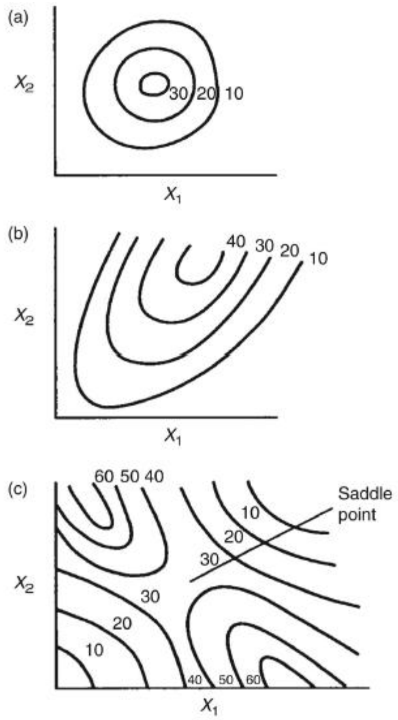


Figure 2– Typical response surface in two dimentions. a) mound, b) raising ridge, c) saddle.

The technique may be applied at different levels of sophistication. Hendrix applied the technique at its simplest level to predict the optimum combination of two variables. The values of the variables for the initial experiments are chosen randomly or with the guidance of previous experience of the process. There is little to be gained from using more than 15–20 experiments. The resulting contour map gives an indication of the area in which the optimum combination of variables resides. A new set of experiments may then be designed within the indicated zone.

The more sophisticated applications of the response surface technique use mathematical models to analyze the first round of experimental data and to predict the relationship between the response and the variables. These calculations then allow predictive contours to

be drawn and facilitate a more rapid optimization with fewer experiments. If three or more variables are to be examined, then several contour maps will have to be constructed.

Check your Progress -2
Note: Write your answer in the space given below
d. Define media optimization.
e. Name the statistical methods of media optimization.
…………………………………………………………………………

3.10 LET US SUM UP

In this unit, you have learnt about the ingredients and raw materials for the industrial media formulations. Uses of agricultural waste, industrial waste by-product as the source/ raw material for the commercially viable compound production has been studied. To know the needs of buffers, additives, pH control agents and antifoams in industrial media. This knowledge would make you understand the basic problems in industrial media formulation for higher productivity. Further, statistical optimization of industrial media and media components also studied. All this content might play very important role in future research.

3.11 UNIT - END EXERCISES

1. Give any for carbon source for media preparation.
2. How agricultural based raw materials used for industrial media?
3. What is pharmamedia?
4. Briefly describe the statistical tools for media optimization.

3.12 ANSWERS TO CHECK YOUR PROGRESS

a) Carbon source, nitrogen source and minerals are basic requirements.
b) Chemotrophs use chemical compounds as a *source* of *energy*.
c) Soyabean meal, yeast extrct, Urea, Ammonium sulphate, etc..
d) Medium optimization is a process where components of medium or different conditions either varied in concentration or changed so that we can get better growth of the organisms for high productivity.
e) Design of experiments (DOE), Response surface *methodology* (RSM), *Plackett-Burman* design.

3.13 SUGGESTED READINGS

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UNIT -IV: STOICHIOMETRY OF CELL GROWTH, STERILIZATION OF MEDIA AND SCALE-UP TECHNOLOGY

Structure

4.1 INTRODUCTION

After going through the unit you will be able to;

- Understand the concept of microbial growth and generation time.
- Know the process of batch, continuous and fed batch culture, biomass production and product synthesis.
- Understand the process of media sterilization.
- Value the scale-up process of media, fermenter and other parameters.

4.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the concept of microbial growth.
- Understand the industrial sterilization methods.
- Understand the process of media sterilization.

- Know the scale-up parameters,
- Value the scale-up parameters for improved product fermentation.

4.3 MICROBIAL GROWTH KINETICS DURING FERMENTATION

Microbial growth is defined as an orderly increase in cell population or the biomass. Microbial growth is occurred in four different ways: bacteria grow by binary fission, yeast divide by budding, and fungi divide by chain elongation and branching. Growth of the cell number or cell mass can be explained quantitatively as a doubling of the cell number per unit time for bacteria and yeasts or a doubling of biomass per unit time for filamentous organisms such as fungi. Growth can be measured by direct counts (Haemocytometer), visually or using instruments, indirect cell counts by turbidity (to use the spectrophotometer to estimate absorbance of cell suspensions), and viable count by colony forming units (CFU). Each viable cell will form a colony and each colony that can be counted is called a colony forming unit (cfu) and the number of cfus is related to the viable count in the sample. Turbidity measurement is a fast and nondestructive method specifically for estimating large numbers of bacteria in clear liquid media. The biochemical activity may also be measured – e.g., O_2 uptake, CO_2 production, ATP production. This method requires a fixed standard to relate chemical activity to cell mass and/or volume.

Bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH etc.) define the bacterium's generation time (G). Generation times for bacteria vary from about 12 minutes to 24 hours. The generation time for *E. coli* in the laboratory is 15-20 min. Bacteria grow exponentially by binary fission, the increase in a bacterial population is by geometric progression. The generation time is the time interval required for cells (or population) to divide: $G = t/n$.

Where G is generation time, n is number of generations and t is time in min/hours

The equation for growth by binary fission is: $x = X \cdot 2^n$

where x is number of bacteria at end of a time interval, X is number of bacteria at beginning of a time interval, n is the number of generations (number of times the population doubles in the time interval).

$$\log x = \log X + n\log 2$$

$$n = \frac{\log x - \log X}{\log 2}$$

$$n = \frac{\log x - \log X}{0.301}$$

$$n = 3.3 \log x/X$$

$$G = \frac{t}{3.3 \log x/X}$$

3.4 GROWTH KINETICS IN BATCH FERMENTATION

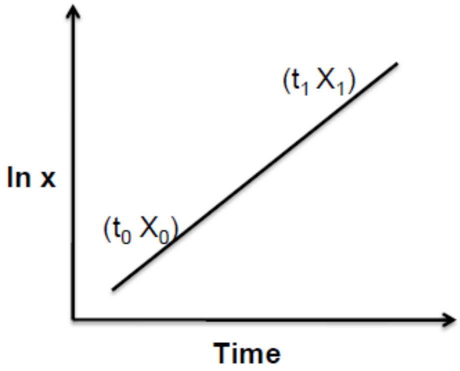
Batch culture occurs in a closed system that contains an initial limited amount of substrate. The inoculated microorganism will pass through a number of growth phases (Fig. 1). During the log phase, cell numbers increase exponentially at a constant maximum rate. In mathematical terms, we can write: $dx / dt = \mu x$

where x is the concentration of microbial biomass, t is the time in hours and μ is the specific growth rate in hours^{-1}. If we integrate between time t_0 and time t_1 when the concentrations of the cells are X_0 and X_1 we obtain: $x_t = x_0 e^{\mu t}$

where x_0 is the original biomass concentration, x_t is the biomass concentration after a time interval (t hours) and e is the base of the natural logarithm. On taking natural logarithms, the equation becomes: $\ln x_t = \ln x_0 + \mu_t$.

Using this equation, a plot of the natural log of biomass concentration versus time should yield a straight line, the slope of which will equal the specific growth rate (μ).

$$\mu = \text{Slope} = \frac{\ln (X_1 - X_0)}{t_1 - t_0}$$



Figur-1: Exponential growth of a bacteria in batch culture.

During the exponential phase, nutrients are in excess and the microorganism is growing at maximum specific growth rate μ^{max} for the prevailing conditions. After a time, the growth rate of the culture ceases. The cessation of growth may be due to the depletion of some essential nutrient in the medium (substrate limitation), the accumulation of some autotoxic product in the medium or a combination of the two. The nature of the limitation of growth can be explored by growing the microorganisms in a range of substrate concentrations and plotting the biomass concentration in the stationary phase against the initial substrate concentration (Figure -2).

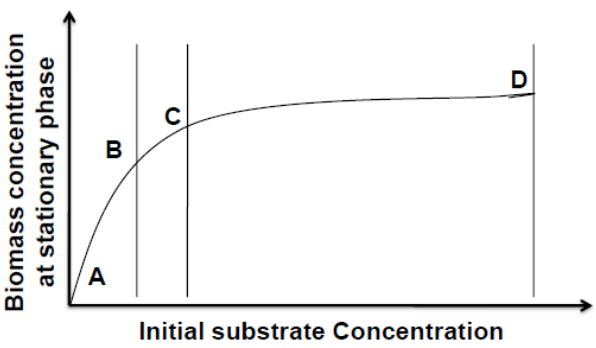


Figure - 2: The effect of initial substrate concentration on the biomass concentration in batch culture stationary phase.

A proportional increase in biomass is observed to increasing initial substrate concentration in the area between A and B which can be defined as: $x = Y (S_R - S)$

Where x is the concentration of biomass produced, Y is the yield factor (g biomass produced per g substrate utilized), S_R is the initial substrate concentration and S is the residual substrate concentration.

In the area between A and B, S is zero and therefore the equation above could be used to predict the biomass that could be formed from a certain amount of substrate. Biomass increases in between B and C with increasing substrate concentration. After that, there is a diminished effect due to accumulation of toxic products or reduced availability of some other substrate (between C and D). However, there is no change in biomass with increasing substrate concentration due to increasing levels of toxic products.

Y, the yield factor is the measure of efficiency of conversion of any one substrate to biomass. Although Y is not a constant and it varies according to growth rate at pH, temperature and the available limiting substrate. The specific growth rate against the concentration of growth-limiting substrate (Fig. 3), describing the relationship between the specific growth rate and the growth limiting substrate concentration is:

$$\mu = \frac{\mu_m S}{K_S + S}$$

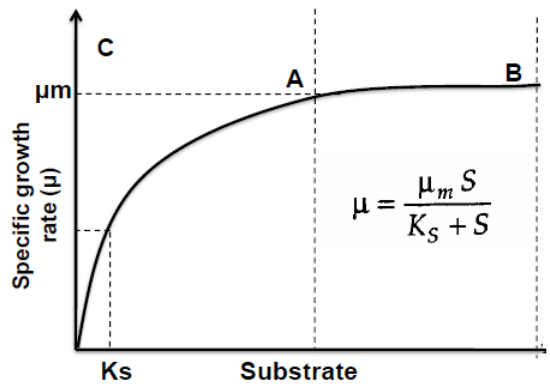


Figure 3: The effect of residual limiting substrate concentration on specific growth rate of a hypothetical bacterium.

Where μ_m is the maximum specific growth rate, S is the residual substrate concentration and Ks is the substrate utilization constant, numerically equal to substrate concentration when μ is half μ_m. Zone A to B (Fig. 3) represents the exponential phase of growth (μ_m) in batch culture where substrate concentration is in excess. Zone A to C is the deceleration phase, substrate concentration becomes limiting and cannot support growth at μ_m. An organism with a high affinity for the limiting substrate (low Ks) will have a short deceleration phase as the growth rate will only be affected when the substrate concentration is very low. In contrast, a microorganism with a low affinity for the

substrate will have a very long deceleration phase (even at high concentration of substrate). Many organisms are producing products during this phase (secondary metabolites).

The metabolic activity of the stationary phase has also been recognized in the physiological descriptions of microbial growth in which the growth of the organism into several phases:

a. The balanced phase: equivalent to the early to middle exponential phase.
b. The storage phase: equivalent to the late exponential phase where the increase in mass is due to the accumulation of lipid and carbohydrate.
c. The maintenance phase: equivalent to the stationary phase.

Monod's model is widely used to describe the growth of many microorganisms. In complex fermentation systems, the following kinetic equation can be used to describe the product expression or product inhibition:

$$\mu = \left[1 - \frac{P}{P_m}\right]^n \frac{\mu_m S}{K_S + S}$$

Using the Monod model, a simple form of microbial growth is written as:

$$\frac{dX}{dt} = \mu X = \frac{\mu_m S}{K_S + S} X$$

$$\frac{dS}{dt} = -\frac{1}{Y_{xs}} \mu X = -\frac{1}{Y_{xs}} \frac{\mu_m S}{K_S + S} X$$

where $\mathbf{Y_{xs}}$ is the biomass yield coefficient. The biomass yield coefficient is the efficiency of conversion of substrate to biomass and is calculated as:

Biomass = Dry weight of biomass produced / Weight of substrate used

The kinetics of product formation may be described as (a) growth-linked products and (b) non-growth linked products. In the first instance – these could relate to primary metabolites synthesized by growing cells and the non-growth-linked products would be secondary metabolites.

Formation of growth-linked products can be defined by the following: **$dp/dt = q_p x$**

where p is the concentration of product, q_p is the specific rate of product formation (mg product /g biomass/h).

Product formation can also be expressed in relation to biomass as: **$dp/dx = Y_{p/x}$**

where $Y_{p/x}$ is the yield of product in terms of biomass (g product/g biomass).

Combining these equations: **$q_p = Y_{p/x} . \mu$**

Thus when product formation is linked to growth, the specific rate of product formation is increases with specific growth rate (**μ_m**). In this instance improved output will be obtained by increasing both biomass and μ.

Non-growth linked product formation is related to biomass concentration. As these products are produced only under certain physiological conditions (usually limitation of a certain substrate), the biomass needs to be in the correct physiological state before secondary metabolites are produced.

Therefore, batch fermentations may be used to produce biomass and primary and secondary metabolites.

(i) Biomass production: conditions supporting fastest growth rate and maximum cell concentration;
(ii) Primary metabolites: conditions to extend exponential phase accompanied by product excretion;
(iii) Secondary metabolites: conditions providing a short exponential phase and extended production phase or conditions giving decreased growth rate in the log phase resulting in earlier secondary metabolite production.

4.5 GROWTH KINETICS IN CONTINUOUS CULTURE

Exponential growth in batch culture may be prolonged by the addition of fresh medium to the vessel. Provided that the medium has been designed such that growth is substrate limited (ie, by some component of the medium), and not toxin limited, exponential growth will proceed until the additional substrate is exhausted. This exercise

may be repeated until the vessel is full. However, if an overflow device was fitted to the fermenter such that the added medium displaced an equal volume of culture from the vessel then continuous production of cells could be achieved If medium is fed continuously to such a culture at a suitable rate, a steady state is achieved eventually, that is, formation of new biomass by the culture is balanced by the loss of cells from the vessel. The flow of medium into the vessel is related to the volume of the vessel by the term dilution rate, *D,* defined as:

$$D = F/V \ (1)$$

where *F* is the flow rate ($dm^3\ h^{-1}$) and *V* is the volume (dm^3).

Thus, *D* is expressed in the unit h^{-1}.

The net change in cell concentration over a time period may be expressed as:

$$dx/\ dt = \text{growth - output}$$

or

$$dx/dt = \mu x - Dx. \ (2)$$

Under steady-state conditions the cell concentration remains constant, thus $dx/dt = 0$ and:

$$\mu x = Dx \ (3)$$

and

$$\mu = D. \ (4)$$

Thus, under steady-state conditions the specific growth rate is controlled by the dilution rate, which is an experimental variable. It will be recalled that under batch culture conditions, an organism will grow at its maximum specific growth rate and, therefore, it is obvious that a continuous culture may be operated only at dilution rates below the maximum specific growth rate. Thus, within certain limits, the dilution rate may be used to control the growth rate of the culture.

The growth of the cells in a continuous culture of this type is controlled by the availability of the growth limiting chemical component of the medium and, thus, the system is described as a chemostat. The mechanism underlying the controlling effect of the dilution rate is essentially the relationship expressed in as:

$$\mu = \mu_{max}\ \check{S}/\ (Ks + \check{S})$$

At steady state, $\mu = D$, and, therefore,

$$D = \mu_{max}\ \check{S}/\ (Ks + \check{S})$$

where Š is the steady-state concentration of substrate in the chemostat, and

$$\check{S} = KsD/(\mu_{max} - D) \quad (5)$$

If substrate is depleted below the level that supports the growth rate dictated by the dilution rate, the following sequence of events takes place:

1. The growth rate of the cells will be less than the dilution rate and they will be washed out of the vessel at a rate greater than they are being produced, resulting in a decrease in biomass concentration.
2. The substrate concentration in the vessel will rise because fewer cells are left in the vessel to consume it.
3. The increased substrate concentration in the vessel will result in the cells growing at a rate greater than the dilution rate and biomass concentration will increase.
4. The steady state will be reestablished.

Thus, a chemostat is a nutrient-limited self-balancing culture system that may be maintained in a steady state over a wide range of submaximum specific growth rates.

The concentration of cells in the chemostat at steady state is described by the equation:

$$\ddot{X} = Y/(S_R - \check{S}) \quad (6)$$

Where, Ẍ is the steady-state cell concentration in the chemostat.

By combining Eqs. (4) and (5), then:

$$\overline{x} = Y\left[S_R - \left\{ \frac{K_s D}{(\mu_{\max} - D)} \right\} \right]$$

Thus, the biomass concentration at steady state is determined by the operational variables, S_R and D. If S_R is increased, Ẍ will increase but Š , the residual substrate concentration in the chemostat at the new steady state, will remain the same. If D is increased, μ will increase ($\mu = D$) and the residual substrate at the new steady state would have increased to support the elevated growth rate; thus, less substrate will be available to be converted into biomass, resulting in a lower biomass steady state value

Check your Progress-1

Note: Write your answer in the space given below

a. Define bacterial growth.

b. Give the formula for steady state growth of a bacteria in continuous fermentation.

……………………………………………………………………………
……………………………………………………………………………
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4.6 STERILIZATION

Industrial fermentation is carried out using suitable production media in the fermenter. The culture media and the culture vessels are frequently contact with different microbes from either water or air. To remove the unnecessary contaminating microbes from the culture media and the vessels before start of fermentation is important. The following are the consequences of such contamination during fermentation:

1. The medium would have to support the growth of both the production organism and the contaminant, resulting in a loss of productivity.
2. If the fermentation is a continuous one then the contaminant may "outgrow" the production organism and displace it from the fermentation and reduces the yield.
3. The contaminant may produce compounds that make subsequent extraction of the final product difficult.
4. The contaminant may degrade the desired product
5. Contamination of a bacterial fermentation with phage could result in the lysis of the culture.

To avoid the contamination:

1. Design and construction suitable fermentation plant.
2. Using a pure inoculum to start the fermentation.
3. Sterilizing the medium and the fermenter vessel.
4. Sterilizing all materials to be added to the fermentation during the process, for example, air, nutrient feeds, antifoams, and pH titrants.
5. Maintaining aseptic conditions during the fermentation.

4.7 MEDIUM STERILIZATION

Industrial media may be sterilized by filtration, radiation, ultrasonic treatment, chemical treatment, or heat. However, for practical reasons, steam is used almost universally for the sterilization of fermentation media except animal-cell culture media.

The destruction of microorganisms by steam (moist heat) may be described as a first-order chemical reaction and, thus, may be represented by the following equation: $-dN/dt = kN$

where N is the number of viable organisms present, t is the time of the sterilization treatment (minutes), k is the reaction rate constant of the reaction, or the specific death rate (min^{-1}). In this case, if we consider the total number of organisms present in the volume of medium, the following equations is use,

$$N_t/N_0 = e^{-kt}$$

where N_0 is the number of viable organisms present at the start of the sterilization treatment, N_t is the number of viable organisms present after a treatment period, t minutes.

On taking natural logarithms

$$\ln\left(\frac{N_t}{N_0}\right) = -kt$$

A plot of the natural logarithm of N_t/N_0 against time yields a straight line, the slope of which equals $-k$. This kinetic description makes two predictions that appear anomalous:

1. An infinite time is required to achieve sterile conditions (ie, $N_t = 0$).
2. After a certain time, there will be less than one viable cell present.

Thus, in this context, a value of N_t of less than one is considered in terms of the probability of an organism surviving the treatment. For example in a particular treatment period reduced the population to 0.1 of a viable organism, this implies that the probability of one organism surviving the treatment is one in ten.

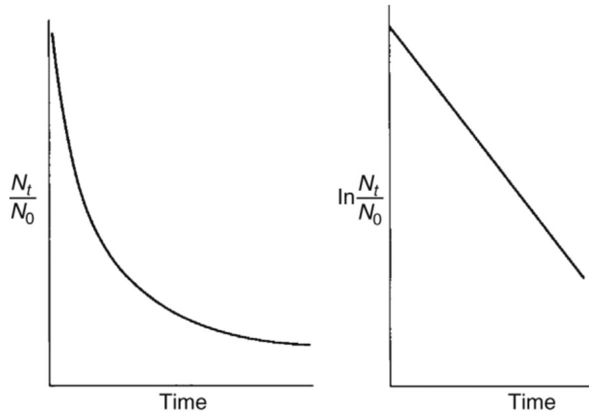


Figure-4: Graph of the proportion of survivors and the Natural Logarithm of the proportion of survivors in a Lethal Temperature at a time period.

Pure culture of organisms undergone the sterilization process, all cells have the same physiological form under ideal sterilization conditions. In a spore forming Bacillus culture, survival of spore varied in different temperature and the time of exposure. The deviation from an immediate exponential decline in viable spore number is due to the heat activation of the spores, that is the induction of spore germination by the heat and moisture of the initial period of the sterilization process. Therefore, viable numbers increase before the observation of exponential decline. However, in the sterilization of mixed cultures containing two species with different heat sensitivities, the initial decline is depends on the population of most heat sensitive and the late decline represented the more resistant populations. .

Thus, a plot of the natural logarithm of the time required to achieve a certain ∇ value against the reciprocal of the absolute temperature will yield a straight line, the slope of which is dependent on the activation energy, as shown in Fig. 5.5. From Fig. 5.5 it is clear that the same degree of sterilization (∇) may be obtained over a wide range of time and temperature regimes; that is, the same degree of sterilization may result from treatment at a high temperature for a short time as from a low temperature for a long time.

When fermentation media can be sterilized by steam under pressure, two types of reaction contribute to the loss of nutrient quality:

1. **Cross reactivity of media components:** A common occurrence during sterilization is the Maillard-type browning reaction that results in discoloration of the medium as well as loss of nutrient quality and the accumulation of growth-inhibitory compounds. Normally it occurs between carbonyl groups, usually from reducing sugars, with the amino groups of amino acids and proteins. For example, glucose in a corn-steep liquor medium. This problems is resolved by sterilizing the sugar separately, after cooling, mix with other sterile nutrient solution.
2. **Degradation of heat labile components***:* Certain vitamins, amino acids, and proteins may be degraded during a steam sterilization method. For example, the preparation of media for animal-cell culture, filtration may be used.

The above said problems may be resolved for most microbial fermentations by the correct choice of steam sterilization method. However, the thermal destruction of essential media components conformed to first order reaction kinetics, as described by the following equation:

$$\mathbf{X_t/X_0 = e^{-kd.t}}$$

Where X_t is the concentration of nutrient after a heat treatment period, t; X_0 is the original concentration of nutrient at the onset of sterilization; kd is the reaction rate constant for the destruction of the medium component.

Thus, the ideal technique would be to sterilize the fermentation medium is heat at a high temperature at a short period. The only practical method of a short-time, high-temperature treatment is to sterilize the medium in a continuous stream. Moreover, the Maillard reactions between medium ingredients can be completely and conveniently, avoided by sterilizing reducing sugars and separately. This is achieved by incorporating several mixing tanks for the different components upstream of the sterilizer and processing the streams through sequential process.

Continuous sterilization also gives saving in the design of the fermenter. If medium is sterilized batch wise in the fermenter, it must be agitated without aeration and it require high capacity motors. The relative merits of batch and continuous sterilization may be summarized as follows:

Advantages of continuous sterilization over batch sterilization:

1. Superior maintenance of medium quality.
2. Ability to sterilize medium components separately.
3. Superior energy efficiency, consuming 60–80% less steam and cooling water.
4. Ease of scale-up—discussed later.
5. Easier automatic control.
6. The reduction of surge capacity for steam.
7. The reduction of sterilization cycle time and hence the reduction in fermenter turnaround time, thus increasing productivity.
8. Under certain circumstances, the reduction of fermenter corrosion.
9. Enables the use of a lower capacity agitator in the fermenter giving economies in both capital and running costs.

Advantages of batch sterilization over continuous sterilization

1. Lower capital equipment costs.
2. Lower risk of contamination—continuous processes require the aseptic transfer of the sterile broth to the sterile vessel.
3. Easier manual control.
4. Easier to use with media containing a high proportion of solid matter.

Batch Sterilization Method

The batch sterilization is a short-time high-temperature method, the medium for a fermentation may be attained either in the fermentation vessel or in a separate container. The major advantages of a separate medium sterilization vessel may be as:

a. Single sterilizer unit may be used to serve several fermenters and the medium may be sterilized as the fermenters are being cleaned and prepared for the next fermentation, thus saving time between fermentations.
b. The medium may be sterilized in a cooker in a more concentrated form than would be used in the fermentation and then diluted in the fermenter with sterile water prior to inoculation.
c. If the medium is most viscous during sterilization and should be diluted with sterile water require less power motor enough for aeration and agitation. Obviously, the sterilization kettle is equipped with a powerful motor.

d. The fermenter would be spared the corrosion that may occur with medium at high temperature.

The major disadvantages of a separate medium sterilization vessel as:

a. The cost of constructing a batch medium sterilizer is much the same as that for the fermenter.
b. If a single sterilizer used for number of fermenters have equipped with complex pipe-work needed to transport the sterile medium, with the intrinsic dangers of contamination.
c. Mechanical failure in a sterilizer supplying medium to several fermenters would affect all the fermenters. The provision of additional equipment may be costly.

4.8 DESIGN OF CONTINUOUS STERILIZATION PROCESS

The design of continuous sterilization cycles may be approached in exactly the same way as for batch sterilization systems. The continuous system includes

a) A time period during which the medium is heated to the sterilization temperature,
b) A holding time at the desired temperature, and
c) A cooling period to restore the medium to the fermentation temperature.

The length of the holding period is dictated by the length of the coil and the flow rate of the medium. The hot medium is then cooled to the fermentation temperature using two sequential heat exchangers. During the process, the nutrient solution in the first heat exchanger is preheated to 90-120^{o}C within 20-30 seconds by the exiting previously sterilized nutrient solution. Then in the second heat exchanger heated the media indirectly with steam to 140^{o}C. This temperature is maintained for 30 to 120 seconds in a holding coil before it is placed in th first exchanger for cooling to the temperature of the fermenter. The cooling phase is also 30-40 seconds. During the process of heat exchangers, 90% of the energy input is recovered. The disadvantage of this method is , some nutrient solutions, insoluble salts (phosphates, oxalates) may formed and crust appear in the first heat exchanger. The extreme temperature

variation in between the sterilized nutrient solution and the cold incoming solution, the heat transfer co efficient is calculated as,

$K = Q / A\text{-}\Delta T_m$

where, K is the heat transfer coefficient; Q is the total heat consumed; A is the transfer surface and ΔT_{m} is the average temperature gradient in the system.

If insoluble precipitant occurs, it will affect the heat exchange/ transfer and the system must be treated with cleaning agents followed with resterilization.

The major advantage of the continuous process is that a much higher temperature may be utilized, thus reducing the holding time and reducing the degree of nutrient degradation. The required Del factor may be achieved by the combination of temperature and holding time that gives an acceptably small degree of nutrient decay. The Del factor for the sterilization is given in the table-1

Table- 1 : Del factor (temp. ^{o}C) and holding time.

Temperature (°C)	**Holding time**
130	2.44 min
135	51.9 s
140	18.9 s
150	2.7 s

i) **Spiral Heat exchanger:** Temperatures steam is passed through one spiral from the center of the exchanger and medium through the other spiral from the outer rim of the exchanger, in countercurrent streams. Spiral heat exchangers are also used to cool the medium after passing through the holding coil. Incoming unsterile medium is used as the cooling agent in the first cooler so that the incoming medium is partially heated before it reaches the sterilizer and, thus, heat is conserved.

The major advantages of the spiral heat exchanger are:

a. The two streams of medium and cooling liquid, or medium and steam, are separated by a continuous stainless steel barrier with gasket seals. This makes cross contamination between the two streams unlikely.

b. The exchanger tends to be self-cleaning which reduces the risk of sedimentation, fouling, and “burning-on.”

ii) Indirect plate heat exchangers: It consists of alternating plates through which the countercurrent streams are circulated. Gaskets separate the plates and failure of these gaskets can cause cross-contamination between the two streams. Also, the clearances between the plates are such that suspended solids in the medium may block the exchanger and, thus, the system is only useful in sterilizing completely soluble media. However, the plate exchanger is more adaptable than the spiral system in that extra plates may be added to increase its capacity.

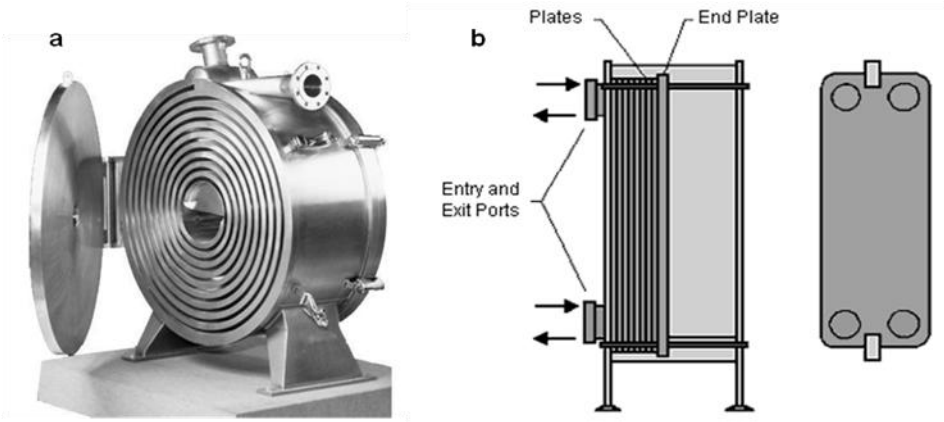


Figure-5: Plate heat exchanger.

The continuous steam injector injects steam directly into the unsterile broth. The advantages are:

a. Very short (almost instantaneous) heating up times.
b. It may be used for media containing suspended solids.
c. Low capital cost.
d. Easy cleaning and maintenance.
e. High steam utilization efficiency.

However, the disadvantages are:

a. Foaming may occur during heating.
b. Direct contact of the medium with steam requires that allowance be made for condense dilution and requires "clean" steam, free from anticorrosion additives.

In some cases the injection system is combined with flash cooling, where the sterilized medium is cooled by passing it through an expansion valve into a vacuum chamber. Cooling then occurs virtually instantly. A flow chart of a continuous sterilization system using direct steam injection is shown in Fig. 6 In some cases a combination of direct and indirect heat exchangers may be used. Example, starch-containing broths.

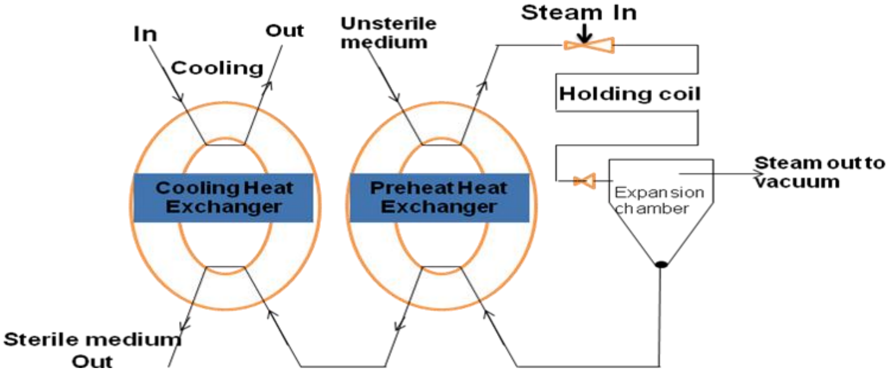


Figure-6: Continuous sterilization of culture media using two types of heat exchangers.

The most widely used continuous sterilization system is that based on the spiral heat exchangers that replaces a pilot-plant direct-steam injection process with one based on indirect spiral heat exchangers. The quality of the medium has been maintained properly. The Del factor to be achieved in a continuous sterilization process has to be increased with an increase in scale. Microorganisms contained within solid particles are given considerable protection against the sterilization treatment. If the residence time in the sterilizer is insufficient for heat to penetrate the particle then the fermentation medium may not be rendered sterile. The routine solution to this problem is to “over design” the process and exposes the medium to a far more sensitive system than may be necessary. The advantage of the continuous process is that temperature may be used as a variable in scaling up a continuous process so that the increased ∇ factor may be achieved while maintaining the nutrient quality constant.

On scaling up the fermentation, it is very difficult to attain the correct sterilization conditions using a batch regime. However, continuous sterilization is a precise process using direct steam injection producing large volume of sterile medium with the required degree of quality. When fermentation is scaled up, it is important to appreciate that the inoculum development process is also increased in scale and a larger seed fermenter may have to be used to generate sufficient inoculum to start the production scale.

4.9 STERILIZATION OF THE FERMENTER

If the medium is sterilized in a separate sterilizer either batch, or is sterilized continuously, then the fermenter has to be sterilized separately before the sterile medium is added to it. This is normally achieved by heating the jacket or coils of the fermenter with steam and sparging steam into the vessel through all entries, apart from the air outlet from which steam is allowed to exit slowly. Steam pressure is held at 15 psi in the vessel for approximately 20 min. It is essential that sterile air is sparged into the fermenter after the cycle is complete and pressure is maintained; otherwise a vacuum may develop and unsterile air be drawn into the vessel.

Sterilization of the Feed Materials

At the time of fermentation, different essential additives may be added to a fermenter and it is in sterile condition. The sterilization method is depends on the nature of the additive, and the volume to be used. When it is require in large quantities then continuous sterilization may be enviable. Feeds could be sterilized sequentially through the sterilizer by separating the different feeds with a plug-flow of water through the system. Filtration is also a commonly used sterilization method for feeds. However, the wet heat sterilization of feeds is the common practice in industries. For moist heat sterilization, the water activity (aw) of a solution is important, it defined as the partial vapor pressure of water (p) in a substance divided by the standard state vapor pressure of water (p0):

$$= a\ pp\ /\mathrm{w}\ 0$$

Thus, as solute concentration increases, so water activity decreases. The *D*-value of *G. stearothermophilus* spores increased 20-fold at water activities of between 0.2 and 0.4. Thus, account must be taken of the effect of water activity on the kinetics of spore death in designing the sterilization regime. For example, the use of a continuous sterilizer for the addition of oil feeds to industrial scale fermenters. Steam sterilization is a wet-heat process and the low water content of oils can result in sterilization being effectively based on dry heat rather than wet, requiring significantly higher holding times, example, dry spores of *B. macerans* in oil had a *D* value one hundred times that of wet spores in the same medium.

4.10 STERILIZATION OF LIQUID WASTE

Large amount of liquid as waste in industries, they possess the process organisms that have been engineered to produce “foreign” products which contains heterologous genes are subjected to strict containment regulations. Thus, waste biomass of such organisms must be sterilized before disposal. Liquid waste sterilization may be achieved by either batch or continuous methods, but the whole process must be carried out under controlled conditions. Batch sterilization involves the sparging of steam into holding tanks, whereas continuous processes would employ the type of heat exchangers that have been used. A holding vessel for the batch sterilization is must be cooled to below 60°C before it is discharged to waste. Based on the kinetic characters of B. *stearothermophilus* the sterilization processes have to be validated.

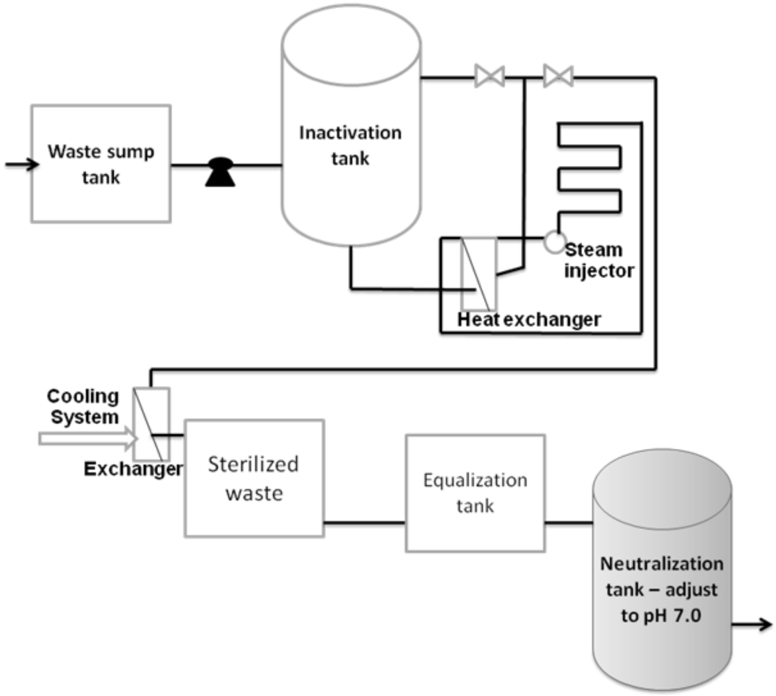


Figure- 7 sterilization of liquid waste from the fermenter using double heat exchangers.

4.12 FILTER STERILIZATION

Filter sterilization methods has been used for sterilization of certain liquids and the gasses. The removal of suspended solids from both gas and liquids may be obtained by the following mechanisms:

1. Inertial impaction: Suspended particles in a fluid stream have momentum. The particles in the suspended fluid will flow through the filter by the route of least resistance. If the particles tend to travel in a straight lines may impacted upon the fibers. Inertial impaction is more significant in the filtration of gases than in the filtration of liquids.

2. Rate of diffusion. Extremely smaller particles in the liquid or air are having random movement and tend to diffuse the filter aids. Diffusion is more significant in the filtration of gases than in the filtration of liquids.

3. Electrostatic attraction*:* Charged particles may be attracted by opposite charges on the surface of the filtration medium.

4. Interception*:* The fibers comprising a filter are assembled to define openings of various sizes. Particles that are larger than the filter pores are removed by direct interception, also some smaller particles are retained by interception. This may be due to more than one particle reached at a pore simultaneously and make a complex bridge to block the pores. Interception is equally important mechanism in the filtration of gases and liquids.

There are two types of filters have been used, i) particles larger than pore size is removed, and ii) particles smaller than the pore size are to be removed. The first type filter is a absolute filter, it 100% efficiently remove the microorganisms. The second type filters are referred as depth filters, basically composed of woven yarns, and loosely packed fiberglass in packed towers in the form of cartridges. Sometime the name absolute and depth filters can be confusing and are named based on its properties, "nonfixed pore filters" (depth filters) and "fixed pore filters" (absolute filters).

i) Nonfixed pore filters: Removal of particles by inertial impaction, diffusion, and electrostatic attraction rather than interception. The packing material contains irregular channels for passing the suspended particles present in the fluid or gas. It is possible that increased pressure applied to a nonfixed pore filter may result in the displacement of previously trapped particles.

ii) Fixed pore filters: The filtration medium will not be distorted during operation (uniform size pore) so that the flow patterns through the filter will not change due to disruption of the material. Some specialized membranes have been used as a filtering aid to trap the suspended particles larger/lesser than the pore size. Fixed pore filters are superior for most purposes such that they have absolute ratings, are less susceptible to changes in pressure, and are less likely to

release trapped particles. The major disadvantage associated with fixed pore filters was the resistance to flow they presented and, hence, the large pressure drop across the filters which represents a major operating cost.

Filtration companies have developed both fixed pore and nonfixed pore filters and the cartridges are then accommodated in stainless steel housings and scale-up can then be achieved conveniently by increasing the number of cartridges.

4.13 STERILIZATION OF FERMENTATION AIR

Majority of the industrial fermentations are operated under vigorous aeration and the air supplied to the fermenter must be sterilized. The number of particles and microorganisms in the air may vary greatly depending on location of the plant, air movement, and previous treatment of the air. The average number of particles in the outdoor air is 10-100000 per m^3 and microorganisms around 5-2500 cells m^{-3}. Among them, 50 % of them are fungal spores and 40% are Gram-negative bacteria.

Fermenters generally work with aeration rate of 0.5 to 1.0 vvm (air volume/ liquid volume*minute. A fermenter having a working volume of 50 m^3 with an aeration rate of 1 vvm needs 3000 m^3 h^{-1}. The method available for sterilizing air includes filtration, gas injection (ozone), gas scrubbing and heat. Of these, only filtration and heat are practical at an industrial scale. Usually the air is passing it over eclectically heated elements, due to high cost this process has been replaced by filtration.

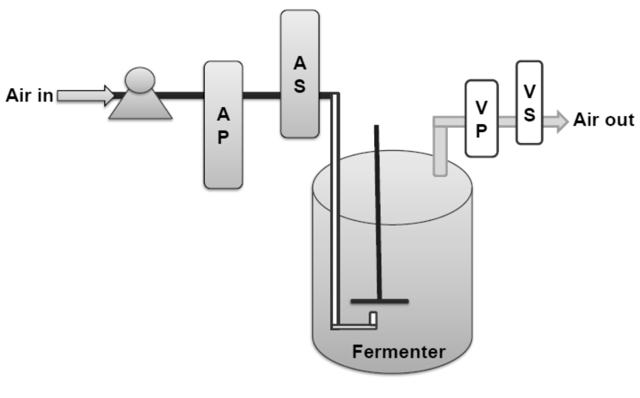


Figure -8 Filter sterilization of gas/air. *AP*, air prefilter; *AS*, air sterilizing filter; *VP*, fermenter vent air prefilter; *VS*, fermenter vent air sterilizing filter

Sterile air can be passes through the depth filters such as glass wool or cotton fibers filter cartridges. The particles would be trapped by a combination of physical effects, including the volume of air, internal effect, blocking effect, diffusion rate, gravity separation, and electrostatic attraction. The disadvantages of the wool filters are shrinkage and solidification during stream sterilization. However, glass fiber filters cartridges; do not have the above problem which can be the better alternative for glass wool filters. New cartridge filters using plated membranes are also available. Membrane pore size is smaller than the wool or activated carbon filters. Operating system is much simpler, because of the removable cartridge construction, replacing the filter elements. Based on the advantageous characters absolute filter for bacteriophages are now industrially in use. Filters of this type (provided they are not physically damaged) are claimed to be 100% efficient in removing microorganisms.

FILTER STERILIZATION OF LIQUIDS

The following liquids are sterilized upstream of the fermenter in a fermentation plant:

- The bulk fermentation medium.
- Nutrient addition feeds, normally comprising one component.
- Water that may be added to a previously sterilized fermenter.
- Acids and alkalis to control pH.
- Buffers—used particularly to control pH during the culture of animal cells. Buffers are also used in the down-stream processing of some parenteral products and have to be sterile.
- Antifoams to control foam formation.

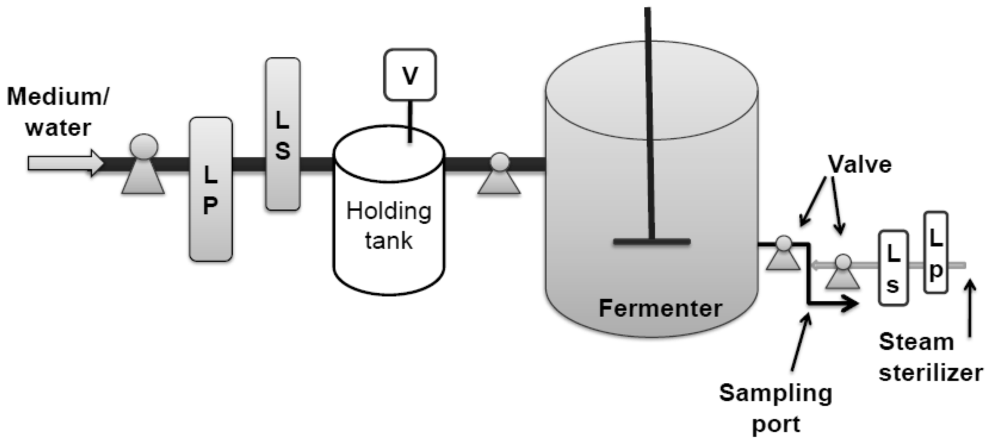


Figure- 9 Filter sterilization of culture medium. *LP*, liquid prefilter; *LS*, liquid sterilizing filter; *V*, holding tank vent filter.

4.14 SCALE-UP OF MICROBIAL FERMENTATION

Microbial fermentation processes play a vital role in many industrial applications. Process scale-up, is a critical activity that enables a fermentation process achieved to operate at a commercially viable scale for manufacturing. A successful scale-up involves the following aspects of successful preparation and planning.

1. Setting up clear goals and expectations,
2. Timelines and milestones,
3. Resources and organization,
4. Facility fit considerations, and
5. Quality and specifications.

The common scale-up project goals includes;

1. Annual output of products, based on market demand and manufacturing capacity
2. Productivity targets (yield/day/lot), based on process capability and facility capability.
3. Process cycle time (days/lot), based on throughput, process, and operational constraints
4. Cost targets, based on process capability, facility operating cost, market demand and product price.

4.14.1 PROCESS SCALE-UP

MASTER CULTURE STRATEGY (Cell banking)

A commercial fermentation process requires a suitable stable cell bank that guarantees consistent and expected production in the long term. A common cell bank strategy for large scale production is to maintain a master cell bank (MCB), from which a working cell bank (WCB) is generated for regular use. This approach allows the original genetically stable MCB to be preserved for an extensive period of time. Most commonly, cell banks are stored in −70°C liquid nitrogen or a −80°C freezer at required quantity. During the storage the quality of the cell bank should be adequately tested and monitored. General tests of a cell bank include:

1. Cell recovery upon freeze and thaw: growth test
2. Contamination or purity tests: based on phenotype tests and genetic discrimination tests, etc.
3. Real-time stability tests: time points for growth and product expression ability test
4. Production qualification: use of a small-scale production model process to check the production

4.14.2 SCALE-UP STRATEGY OF INOCULUM DEVELOPMENT [seed train & inoculums train]

Inoculum development is an important step for microbial fermaentation. The quantity and the quality of inoculums from the seed culture is to propagate cells to a desired mass for inoculation into the bioreactor. The traditional seed train includes thawing a vial and inoculating into shaker flasks for a certain number of stages with increasing flask sizes, and may include stainless steel reactors.

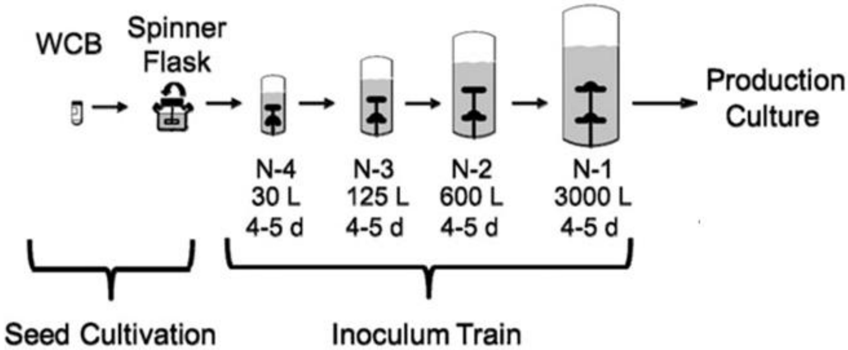


Figure-10: Seed train and inoculums train

Typical studies of seed train scale-up may include:

1. Vial thaw conditions: This may include temperature and time between thaw and inoculation.
2. Inoculation ratio*:* It is always desirable, usually, 0.1 to 5% (v) is used.
3. Seed train media: Usually, the optimized media support to growth rather than product fermentation is used.
4. Genetic stability and production stability during the seed train process.

4.14.3 PRODUCTION FERMENTATION SCALE-UP

The importance of scale-up of a fermentation process is to demonstrate fermentation production at large scale resulting in the same productivity and quality as that developed at small scale. The scale-up fermentation has to be demonstrated by a suitable statistical model with significant consistency. the following are the important steps;

1. Identify linear scale-up parameters.

1. Temperature*:* Temperature should be maintained the same at all scales. Temperatures of microbial processes range widely, mostly from 20 to 45°C. For large scale, consideration should be given to the mixing time, heat transfer rates, and control precision. To maintain temperature, a large-scale bioreactor needs to provide the same heat transfer rate (heat/volume/time) as the small-scale bioreactor does throughout the entire course of a process. Many high-cell-densities cultures require extremely high heat removal step.
2. pH: pH provides an important cell physiological condition. Automated pH control is needed to control pH change in the larger bioreactors. This may be controlled by automatic controller based acid/base delivery system (pump, pressure, valves, etc.).
3. Pressure: The pressure of fermentation may remain the same across scales. It may be necessary to adjust higher hydrostatic pressure at large scale. Pressure may also be adjusted as a strategy or tool to help improve gas transfer at larger scales, where a high agitation rate is difficult to reach.
4. Dissolved-oxygen *(DO)* level: To keep the culture in the same respiration condition, the DO set point is kept consistent across scales. An automated DO control mechanism for a large-scale bioreactor should be required.

5. Airflow rate: Usually, the airflow rate is scaled up by a constant volumetric flow rate per fermentation broth volume. For example, a 1-liter fermentation with an airflow rate of 0.2 liter/min, this is 0.2 vvm. At the 100-liter scale, the flow rate would be 20 liter/min to maintain 0.2 vvm. To obtain an accurate airflow a mass flow device is needed. The success of scale-up is conducted with superficial gas velocity (gas flow per unit cross-sectional area of reactor) kept constant.
6. Final fermentation volume and initial (starting) batch volume: Usually, the ratio of the final fermentation volume versus the initial batch volume is kept constant in scale-up. The final fermentation volume is usually about 70% of the total vessel volume.
7. Nutrient concentrations: Nutrient concentrations are maintained at large scale. The batch medium volume is scaled up proportionally. For fed-batch processes, nutrient feeding rates can be calculated based on the initial batch medium volume.

4.14.4 IDENTIFY NONLINEAR SCALE-UP PARAMETERS.

a. **Agitation**: This parameter is related to mixing time and oxygen transfer.

b. **Nonlinear feeding rates***:* Often glucose and some nutrients are fed exponentially to match the cell growth profile. It is assumed that the exponential feeding of nutrient feed allows the nutrient concentration or uptake rate for each cell to be constant.

4.14.5 PERFORM FERMENTATION PREPARATION SCALE-UP.

a. **Media preparation***:* At large scale, media preparation procedure, vessels, and conditions must be defined. Large-scale media preparation may be a challenge because of the large amounts of materials required with desirable quality like solubility and stability and also the relatively longer hold time prior to sterilization and inoculation. Commonly, components that are sensitive to heat degradation are filter sterilized and components that are not sensitive to heat degradation are heat sterilized for large-scale fermentation.

b. **Media hold**: Certain media will be hold for a short duration prior to feed during the fermentation process.

c. **Acid and base for pH control***:* To identify the types of acid and base that are suitable for large-scale fermentation. Large-scale acid/base vessels are not always suitable for various types of acid

and base used in small-scale studies. The acid and base storage vessels should be corrosion free.

d. **Medium and acid/base delivery systems**: It should be controlled by automated valves and pressure or pump systems are used for the delivery of nutrient feeds, acid, and base. The control of the flow of media and others should be kinetically calculated and regulated.

FERMENTOR SCALE-UP

Bioreactor design scale-up is based on operational parameters such as agitation, airflow rate, and cooling capability based on small-scale data. These parameters facilitate the bioreactor's ability to provide a well-mixed nutrient environment, maintain dissolved oxygen for the requirement of the organism's growth and product fermentation.

The following are the basic parameters should be considered for large scale operation:

1. Constant agitation power input (power/volume)
2. Constant oxygen transfer coefficient (*kLa*) (h^{-1})
3. Constant mixing time (s)
4. Constant agitation impeller tip speed (m/s)
5. Constant heat transfer rate (heat/volume)
6. Constant gas volumetric flow rate (vvm)
7. Constant gas superficial velocity (m/s)

Scale-up process cannot be done with keeping these entire parameters constant, at the same time it should be varied due to the bioreactor's physical limitation. Based on the studies of pilot scale operation, industrial scale design has made.

A fermentor's agitation power input value (power consumption/volume) drives oxygen transfer and mixing time, so it is a simple and practical way to evaluate scale-up.

$$P = Np0pn3dI^5 \quad \text{or} \quad Np0 = P/pn3dI^5$$

where P is power input (kg m^2 s^{-2} = W), Np0 is a dimensionless power number, n is agitation speed, p is density of the medium, and dI is impeller diameter.

Scale-up of microbial fermentation is usually carried out by assessing the adequate oxygen transfer rate (OTR) at a large scale. It depends on agitation speed, airflow rate, pressure, and pure oxygen supply.

$$\mathbf{OTR} = \boldsymbol{kLa(C0 - C)}$$

where *C*0 (mM) is the saturation concentration of oxygen in fermentation broth, influenced by the oxygen content in the inlet airflow and vessel pressure; *C* (mM) is the DO concentration of a culture at a given time; and *kLa* (h^{-1}) is the oxygen transfer coefficient, which is directly related to the fermentor's configuration, agitation speed, impeller design, and airflow rate.

Therefore, *kLa* is a good indicator of a fermentor's oxygen transfer capability. In general, OTRs decrease as fermentor scales increase. The maximum OTRs (in mM O_2/liter/h) are 300 for a bench fermentor, 100 to 150 for a pilot fermentor (>500-liter volume), and 50 to 100 for a large-scale fermentor (>10,000-liter volume).

High-density microbial fermentation usually generated a significant amount of heat. Hence, cooling capacity is important at large-scale production. The ratio of jacket surface area HTR) is a measure of the cooling capability of a fermentor.

$$\mathbf{HTR} = \boldsymbol{hA(T - Tj)}$$

where *h* is the heat transfer coefficient, related to the specific heat transfer efficiency of the jacket material and configuration; *A* is the vessel jacket area; *T* is the culture temperature; and *Tj* is the temperature of coolant inside the jacket.

For the industrial fermenters low coolant temperature and large jacket area will improve heat transfer capability. To guarantee a successful large-scale manufacturing process, heat transfer ratio data must be collected during process optimization.

4.15 VALIDATION OF SCALE-UP

For a fermentation scale-up, the following parameters can be used to evaluate if the scale-up is a success.

1. Product formation rate
2. Cell growth profile
3. Operational aspects
4. Product quality

PRODUCT QUALITY

Product quality is based on the design parameters, scaling up a process may result in product quality differences. Large-scale

fermentation may provide a different environment for microbial cells compared to small-scale fermentation. The differences are usually related to non homogeneous environments at large scale. At large scale, mixing time, gas distribution, nutrient concentration, and temperature distribution may not precisely match those at small scale. These conditions may affect product formation or transport in microbial cells. Therefore, it is prudent to adequately analyze product quality during process scale-up.

WASTE STREAMS AND TREATMENT

For a given scale up process waste outputs will also be increased. Hence, it must be carefully calculated and considered for a design of waste treatment plant. The waste output can be calculated based on material throughput at each step of the process as detailed in the process description. The required treatment of each waste should be evaluated according to environmental regulations. In many cases, biological waste materials including organisms may present potential hazards to the environment. Therefore a suitable waste collection tank and a treatment system consisting of chemical or heat process often are required as large facilities.

CONCLUSION

Though the various diversity of microorganisms and products in large scale fermentation, required a suitable and ‘one-size-fits-all’ strategy for scale-up is not advisable. However, the general and basic principle of scale-up of microbial fermentation has been discussed in this chapter. Scale-up activity is complex, including cell biological factors, nutrient and metabolic factors, and processing related factors. In addition, its success is highly dependent on product quality requirements and project emphasis. Therefore, approaches and models in scale-up may vary due to project emphasis. The keys for scale-up are to achieve project goals, produce desired product with acceptable quality and yield, and fully understand and control operational risks at large-scale manufacturing.

Check your Progress -2

Note: Write your answer in the space given below

c. Define scale-up process.

d. List out the media scale-up stages

e. What are the parameters to be scale-up for industrial fermenter design?

………………………………………………………………………
…………………………………………………………

4.16 LET US SUM UP

In this unit, you have learnt about the microbial growth in batch fermentation, generation time, growth kinetics in batch system, fed-batch system and continuous fermentation system. The sterilization concept for industrial media, fermenter, other apparatus, raw materials, and the waste disposal were discussed elaborately. The concept such as scale-up, scale-up parameters, and the steps taken for scale-up process of industrial scale fermentation process. The information relates this unit helps us to understand the process scale up from laboratory level to larger bioprocess industries.

4.17 UNIT - END EXERCISES

1. What are the methods used for analyse the growth of a bacteria.
2. Discuss the importance of industrial sterilization.
3. Write short note of media scale-up process.

4.18 ANSWERS TO CHECK YOUR PROGRESS

a. Bacterial growth is proliferation of bacterium into two daughter cells, in a process called binary fission. Providing no event occurs, the resulting daughter cells are genetically identical to the original cell. Hence, bacterial growth occurs in four different phases.

b. Steady state growth = $$\bar{x} = Y\left[S_R - \left\{\frac{K_s D}{(\mu_{\max} - D)}\right\}\right]$$

c) Act of using results obtained from laboratory studies for designing a prototype and a pilot plant **process**;construction a pilot plant and using pilot plant data for designing and constructing a full **scale** plant or modifying an existing plant.

d) Media preparation, media hold, pH control, nutrient content and chemical complexity of the raw materials used.

e) Fermenter, strain used, media, process parameters

41.19 SUGGESTED READINGS

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UNIT -V: DESIGN OF A FERMENTOR - I

Structure

5.1 INTRODUCTION

A fermenter is an enclosed and sterile vessel that maintains optimal conditions for the growth of a microorganism. The microorganism undergoes fermentation to produce large quantities of a desired metabolite for commercial use. In this chapter, basic design of a microbial fermenter, need for the quality fermenter, types of fermentation vessels, aseptic operations and containments are to be discuss in elaborate manner.

5.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the concept of a fermenter vessel
- Relate the importance of an ideal fermenter.
- Know the various types of a fermenter.
- Understand the concept of aseptic operation and containments.

5.3 BASIC DESIGN OF A MICROBIAL FERMENTER

The fermenter is used to provide a controlled environment for the growth of microorganisms or animal cells, to obtain a desired product. In designing and constructing a fermenter, a number of points must be considered:

1. The vessel should be capable of being operated aseptically for a number of days and should be reliable in long-term operation and meet the requirements of containment regulations.
2. Adequate aeration and agitation should be provided to meet the metabolic requirements of the microorganism. However, mixing

should not cause damage to the organism nor cause excessive foam generation.

3. Power consumption should be as low as possible.
4. A system of temperature control, both during sterilization and fermentation, should be provided.
5. A system of pH monitoring and control should be provided together with the monitoring and control of other parameters (eg, dissolved oxygen, redox, etc.) as appropriate.
6. Sampling facilities should be provided.
7. Evaporation losses from the fermenter should not be excessive.
8. The vessel should be designed to require the minimal use of labor in operation, harvesting, cleaning, and maintenance.
9. Ideally the vessel should be suitable for a range of processes, but this may be restricted because of containment regulations.
10. The vessel should be constructed to ensure smooth internal surfaces, using welds instead of flange joints whenever possible.
11. The vessel should be of similar geometry to both smaller and larger vessels in the pilot plant or plant to facilitate scale-up.
12. The cheapest materials, which enable satisfactory results to be achieved should be used.
13. There should be adequate service provisions for individual plants (Table 1).

Fermenter involves cooperation between experts in microbiology, biochemistry, chemical engineering, mechanical engineering, and costing. Although many different types of fermenter have been described in the literature, very few have proved to be satisfactory for industrial aerobic fermentations. The most commonly used ones are based on a stirred upright cylinder with sparger aeration. This type of vessel can be produced in a range of sizes from 1 dm^3 to 1000s of dm^3. Figs. 1 and 2 are diagrams of typical mechanically agitated and aerated fermenters with one and three multibladed impellers, respectively. Tables 2 and 3 give geometrical ratios of various of the dimensions which have been quoted in the literature for a variety of sizes of vessel. Moucha, Rejl, Kordac, and Labik (2012) have described the design of multiple-impeller fermenters with particular emphasis on scale-up, oxygen mass transfer, and the limitations of experimental k_La data.

NOTES

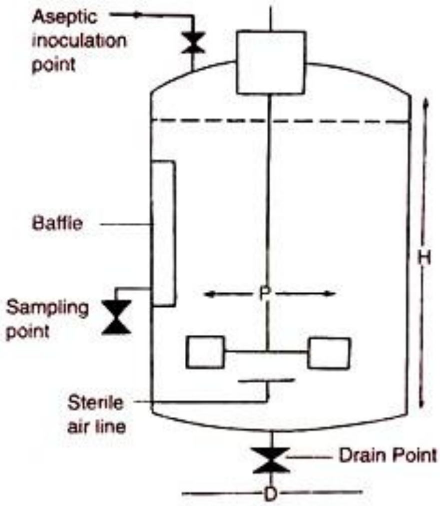


Figure-1: Basic design of a microbial fermenter (agitator).

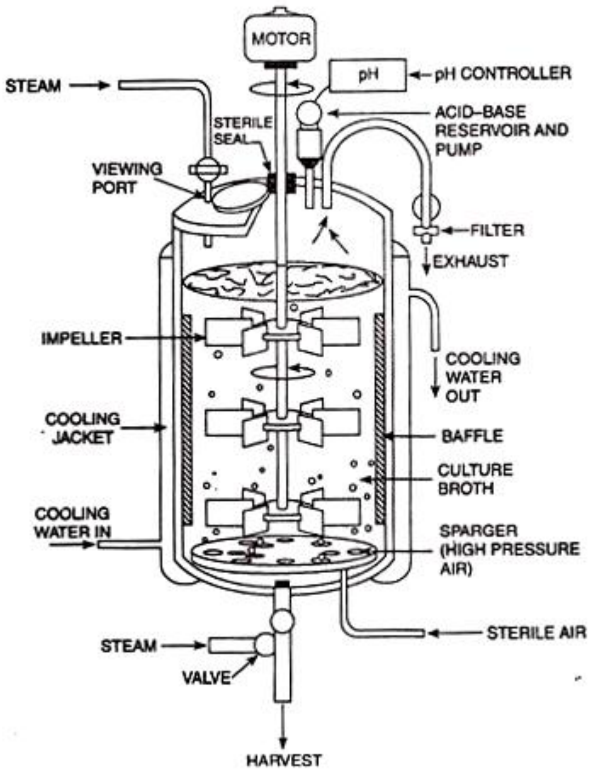


Figure-2: Multiple impeller fermenter.

At this stage the discussion will be concerned with stirred, aerated vessels for microbial cell culture. More varied shapes are commonly used for alcohol, biomass production, animal cell culture, and effluent treatment.

Table 1 Service Provisions for a Fermentation Plant.

S. No.	Service Provisions for a Fermentation Plant
1.	Compressed air
2.	Sterile compressed air (at 1.5–3.0 atm)
3.	Chilled water (12–15°C)
4.	Cold water (4°C)
5.	Hot water
6.	Steam (high pressure)
7.	Steam condensate
8.	Electricity
9.	Stand-by generator
10.	Drainage of effluents
11.	Motors
12.	Storage facilities for media components
13.	Control and monitoring equipment for fermenters
14.	Maintenance facilities
15.	Extraction and recovery equipment
16.	Accessibility for delivery of materials
17.	Appropriate containment facilities

Table 2 Details of geometrical ratios of fermenters with single multiblade Impellers (Fig. 1)

Dimension	Steel and Maxon (1961)	Wegrich and Shurter (1953)	Blakeborough (1967)
Operating volume	250 dm^3	12 dm^3	—
Liquid height (L)	55 cm	27 cm	—
L/D (tank diameter)	0.72	1.1	1.0–1.5
Impeller diameter (P/D)	0.4	0.5	0.33
Baffle width/D	0.10	0.08	0.08–0.10
Impeller height/D	—	—	0.33

5.4 TYPES OF FERMENTATION VESSELS

Fermenter vessels have more limited applications and have been developed for specific purposes or closely related processes. Bioreactors can be classified according to various different criteria,

1. Type and form of biocatalyst: free cells in submerged cultures; carried bound or immobilized cells/enzymes; retention or recirculation of the biocatalyst
2. Configuration: tank (height/diameter <3), column (height/ diameter > 3)
3. Energy input and aeration: liquid phase; gas phase; combined
4. Hydrodynamics: perfect mixing; partial mixing; no mixing;
5. Mode of operation: batch; continuous; fed-batch.

Few of the bioreactor types are discussed below:

5.4.1 STIRRED TANK BIOREACTORS (STB)

One of the most conventional bioreactors is the stirred-tank bioreactor. Fig. 1 & 2 shows a schematic diagram of a typical stirred-tank bioreactor. The core component of the stirred tank bioreactor is the agitator or impeller, which performs a wide range of functions: heat and mass transfer, aeration, and mixing for homogenization. Two types of impellers are widely used in the conventional fermentation industry: axial and radial flow impellers. Impellers are used for: oxygen transfer, heat transfer, power consumption, and fluid dynamics. These greatly facilitate the design, installation and optimization of these impellers in conventional fermentation, and so the standard stirred-tank bioreactor is used almost universally in the fermentation industry. Besides the impeller type, there are a number of geometric specifications important for the performance of the stirred tank reactor; these include

1. the impeller off-bottom clearance,
2. the impeller size,
3. the baffles and their width,
4. the sparger type and position,
5. the ratio of liquid height to tank diameter.

For large-scale vessels, multiple impellers are often been installed in order to provide sufficient mixing and mass transfer.

For shear-sensitive biological systems, such as animal and plant cell cultures, conventional impellers that produce high shear stress cannot be directly applied. Because of their fragile cellular structure, animal cells are very sensitive to shear and bubble damage in the bioreactor environment. The two principal mechanisms that can lead to physical cell damage are hydrodynamic shear force induced by agitation, and air bubble damage caused by unprotected gas sparging.

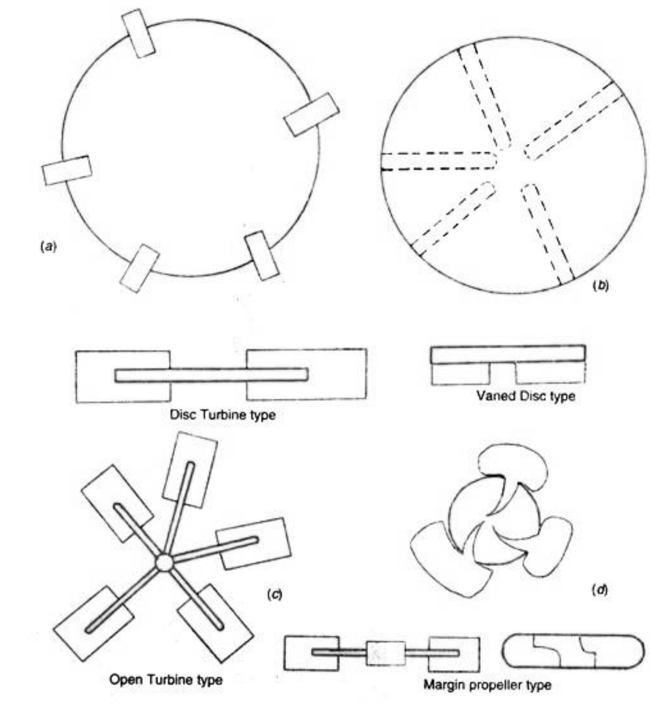


Figure-3: Impellor types in Stirrer tank fermenter.

Along with the installation of a proper oxygenation device, such as bubble-free aeration, gas basket, and cage-aeration, and the addition of appropriate protective agents, many modifications of the marine impeller have been proposed in order to provide more efficient mixing at lower impeller tip speeds. A number of highflow, low-power-number impellers such as Intermig, Lightnin, Prochem Maxflow T and Scaba 6SRGT have been developed to provide improved performance.

Plant cells are also sensitive to shear stress, but not as much as animal cells are. As such, many of the stirred tank bioreactor systems used for plant cell suspension cultures are modifications of those used for microbial systems. The impellers used for plant cell cultures range from the standard Ruston turbine, curved-blade disk turbine, and hydrofoil impellers to semiconventional agitators, such as helical ribbon and centrifugal impellers.

In general, the stirred tank bioreactor has several advantages for the cultivation of shear-sensitive cells:

1. existing industrial capacity,
2. proven performance,
3. ease of maintaining homogeneous conditions, and
4. ease of scale-up and control.

Thus, currently in the biopharmaceutical industry, the stirred-tanks are the most widely used bioreactors for GMP production of monoclonal antibodies (MAb) therapeutics and other biologicals using animal cell cultures. Several biopharmaceutical manufacturers have implemented stirred tank bioreactors at the 10,000 to 20,000 liter scale for large-scale animal cell cultures.

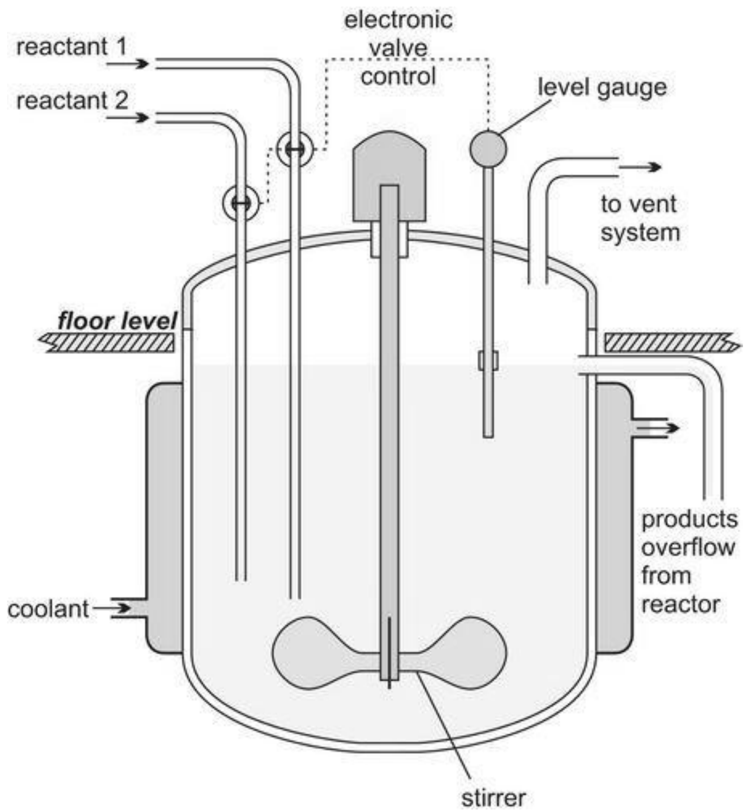


Figure-4: Continuous stirred-tank reactor.

The **continuous stirred-tank reactor** (**CSTR**), also known as vat- or backmix reactor, or a continuous-*flow* stirred-tank reactor (**C*F*STR**), is a common model for a chemical reactor in chemical engineering. A CSTR often refers to a model used to estimate the key unit operation variables when using a continuous agitated-tank reactor to reach a specified output. The mathematical model works for all fluids: liquids, gases, and slurries (Fig.4).

The behavior of a CSTR is often approximated or modeled by that of a Continuous Ideally Stirred-Tank Reactor (CISTR). All calculations performed with CISTRs assume perfect mixing. In a perfectly mixed reactor, the output composition is identical to composition of the material inside the reactor, which is a function of

residence time and rate of reaction. If the residence time is 5-10 times the mixing time, this approximation is valid for engineering purposes. The CISTR model is often used to simplify engineering calculations and can be used to describe research reactors. In practice it can only be approached, in particular in industrial size reactors

Application

Continuous flow stirred-tank reactors are usually applied in waste water treatment processes. CSTRs facilitate rapid dilution rates which make them resistant to both high pH and low pH volatile fatty acid wastes. CSTRs are less efficient compared to other types of reactors as they require larger reactor volumes to achieve the same reaction rate as other reactor models such as PFRPlug Flow Reactors.

5.4.2 AIRLIFT BIOREACTOR

Air-lift fermenter is an efficient contactor for the reactions involved gases, liquids and solids. There are two types of air-lift fermenters which are the internal loop and the external loop. The internal loop has a draft tube in its inner tube, in which the up-flowing gasses liquid and the down-flowing liquid is separated by the draft tube. Draft tubes are used in some processes to promote better mass transfer, mixing and inducing circulatory motion to reduce bubble coalescence. The external loop has two streams flow in two separate pipes connected at top and bottom. In this way, the air-lift fermenters improve the circulation and oxygen transfer and equalize shear forces in the reactor. Both of the internal and external loop air-lift fermenters have been investigated to the hydrodynamic behaviour and other design factors. The figure below shows the structure of an air-lift bioreactor with an internal loop.

Unlike the mechanical agitation system, air-lift fermenters do not have motor, shaft and impeller blades. As such, the mixture inside the fermenters is agitated by injecting air from the bottom of the tube. Sterile atmospheric air is injected into the fermenters if the microorganism is aerobic, while for the anaerobic microorganism is fed with inert gas.

Airlift bioreactors (ALB) are generally classified as pneumatic reactors without any mechanical stirring arrangements for mixing and use the expansion of compressed gas to bring about the mixing. The turbulence caused by the fluid flow ensures adequate mixing of the liquid. The draft tube is provided in the central section of the reactor.

The introduction of the fluid (air/liquid) causes upward motion and results in circulatory flow in the entire reactor. Even large fermenters doesn't require internal cooling coils as a jacket can normally provide sufficient heat transfer, due to the rapid movement of fluid within the vessel. The air/liquid velocities will be low and hence the energy consumption is also low. ALBs can be used for both free and immobilized cells. There are very few reports on ALBs for metabolite production.

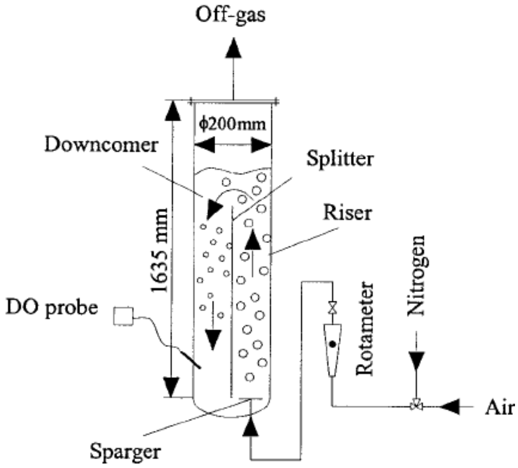


Figure – 5: Split-cylinder Air-lift bioreactor.

There are two types of air-lift fermenters which are the internal loop and the external loop.

1. The internal loop has a draft tube in its inner tube, in which the up-flowing gasses liquid and the down-flowing liquid is separated by the draft tube. Draft tubes are used in some processes to promote better mass transfer, mixing and inducing circulatory motion to reduce bubble coalescence.
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Both of the internal and external loop air-lift fermenters have been investigated to the hydrodynamic behaviour and other design factors **(**Fermentation,

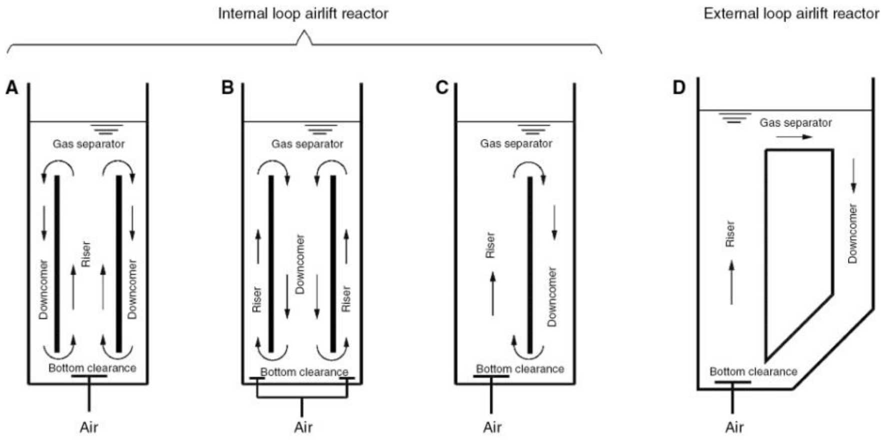


Figure-6: Internal and external loop air-lift reactor.

Mixing in air-lift fermenters is very gentle hence it is suitable for batch culture of shear sensitive cells and tissues such as the mammalian and plant cells. Likewise, high shearing stress causes damage to cells could be avoided. Batch culture of plant and animal cells can be cost intensive. However, in cases where the demand for the plant or animals culture products is low and batch cycles are long, the high capital cost can exclude the economical production. One of the application of air-lift fermenters is the large scale production of monoclonal antibodies.

The advantages of Airlift reactors are the elimination of attrition effects generally encountered in mechanical agitated reactors. It is ideally suited for aerobic cultures since oxygen mass transfer coefficient are quite high in comparison to stirred tank reactors.

5.4.3 Fluidized Bed Bioreactors (FBB)

Fluidized bed bioreactors (FBB) have received increased attention in the recent years due to their advantages over other types of reactors.
Most of the FBBs developed for biological systems involving cells as biocatalysts are three phase systems (solid, liquid & gas). The FBBs are generally operated in co-current upflow with liquid as continuous phase and other more unusual configurations like the inverse three phase fluidized bed or gas solid fluidized bed are not of much importance. Usually fluidization is obtained either by external liquid recirculation or by gas fed to the reactor. In the case of immobilized enzymes the usual situation is of two-phase systems involving solid and liquid but the use of aerobic biocatalyst necessitate introduction of gas (air) as the third phase.

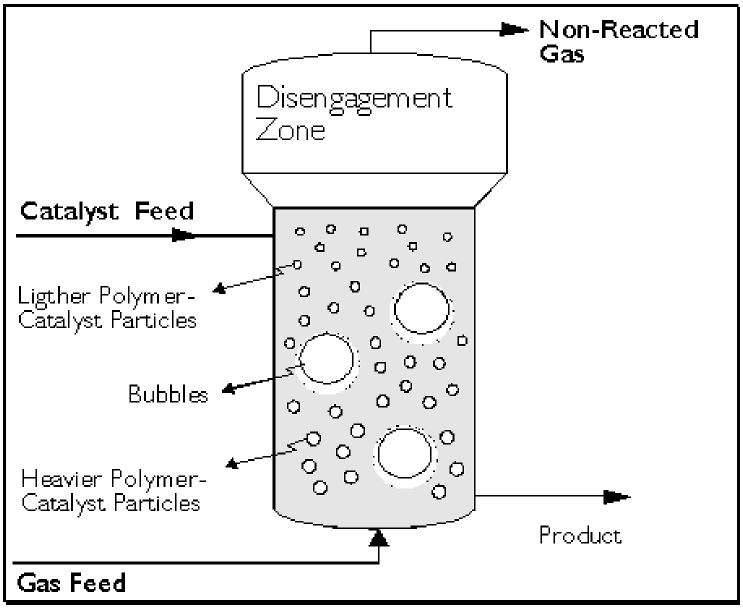


Figure-7: Fluidized bed bioreactor.

A differentiation between the three phase fluidized bed and the airlift bioreactor would be made on the basis that the latter have a physical internal arrangement (draft tube), which provides aerating and non-aerating zones. The circulatory motion of the liquid is induced due to the draft tube. Basically the particles used in FBBs can be of three different types:

1. Inert core on which the biomass is created by cell attachment
2. Porous particles in which the biocatalyst is entrapped.
3. Cell aggregates/ flocs (self-immobilization).

In comparison to conventional mechanically stirred reactors, FBBs provide a much lower attrition of solid particles. The biocatalyst concentration can significantly be higher and washout limitations of free cell systems can be overcome. In comparison to packed bed reactors FBBs can be operated with smaller size particles without the drawbacks of clogging, high liquid pressure drop, channeling and bed compaction. The smaller particle size facilitates higher mass transfer rates and better mixing. The volumetric productivity attained in FBBs is usually higher than in stirred tank and packed bed bioreactors. There are several successful examples of using FBBs in bioprocess development.

5.4.4 PACKED BED BIOREACTORS

Packed bed or fixed bed bioreactors (fig.8) are commonly used with attached biofilms especially in wastewater engineering. The use of packed bed reactors gained importance after the potential of whole cell immobilization technique has been demonstrated. The immobilized biocatalyst is packed in the column and fed with nutrients either from top or from bottom. One of the disadvantages of packed beds is the changed flow characteristic due to alterations in the bed porosity during operation. While working with soft gels like alginates, carragenan etc. the bed compaction which generally occurs during fermentation results in high pressure drop across the bed. In many cases the bed compaction was so severe that the gel integrity was severely hampered. In addition channeling may occur due to turbulence in the bed. Though packed beds belong to the class of plug flow reactors in which backmixing is absent in many of the packed beds slight amount of backmixing occurs which changes the characteristics of fermentation.

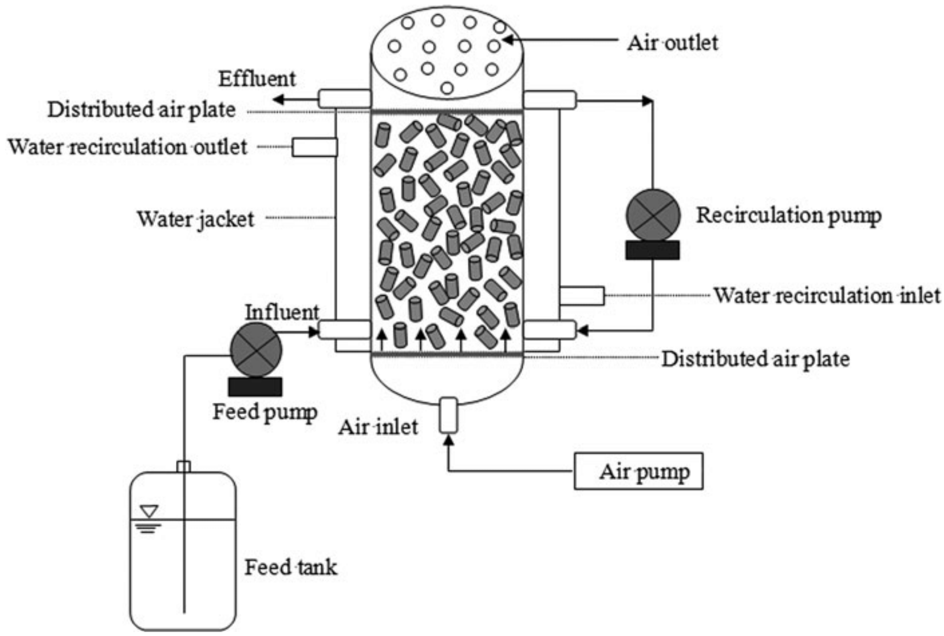


Figure-8: packed bed bioreactor.

Packed beds are generally used where substrate inhibition governs the rate of reaction. The packed bed reactors are widely used with immobilized cells. Several modifications such as tapered beds to reduce the pressure drop across the length of the reactor, inclined bed, horizontal bed, rotary horizontal reactors have been tried with limited success.

5.4.5 **BUBBLE COLUMN REACTOR**

A bubble column reactor is an apparatus used to generate and control gas-liquid chemical reactions. Bubble columns are cylindrical vessels with a gas inlet at the bottom. The gas is sparred in the form of bubbles into a liquid or a liquid–solid suspension. These reactors are generally referred to as slurry bubble column reactors when a solid phase exists

The bubble column reactors (fig. 9) represent contactors in which a gas or a mixture of gases is distributed in the liquid at the column bottom by an appropriate distributor and moves upwards in the form of bubbles causing intense mixing of the liquid phase. The aim of a bubble column is to control the rate of mass transfer and reaction between a gas and a liquid. In general, both liquid and gas are fed continuously in a countercurrent or parallel flow style.

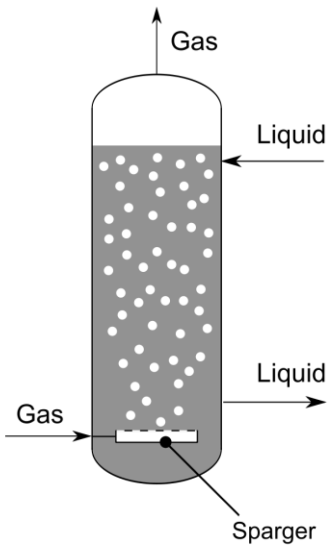


Figure 9. Basic bubble column reactor

A large quantity of the gas is unsuitable for the bubble column because the pressure drop due to the gas flow becomes fairly large. However, absorption controlled by the liquid phase is suitable for a bubble column. The mixing performance of the bubble column has been discussed based on the change in concentration of the injected tracer gas with time at some specific position in the column. Although the bubble column has been widely used, a detailed discussion on its mixing performance remains incomplete. Therefore, it is significant to clarify the relationship among the mixedness, time, and gas flow rate in a bubble column. Bubble column reactors are used in various types

of chemical reactions like wet oxidation, or as algae bioreactor. Since the computerized design of bubble columns is restricted to a few partial processes, experience in the choice of a particular type column still plays an important role.

Various configurations of bubble columns have evolved based on the requirements of efficient contacting bubbles, redistribution of bubbles, suspension and circulation of solids and so on. Some of these variants are shown schematically in Fig. 10. Bubble column reactors are commonly used with internal cooling or heating coils for effective heat management. Multistage or sectionalized bubble column reactors are used in cases where axial backmixing needs to be controlled. Packed bubble column reactors are also used in practice. Bubble column reactors are usually operated in a continuous mode. There are also several variants of bubble columns, such as external or internal loop reactors and jet loop reactors (with gas eductors). Jet loop reactors are typically downflow bubble column reactors and offer excellent gas-liquid performance.

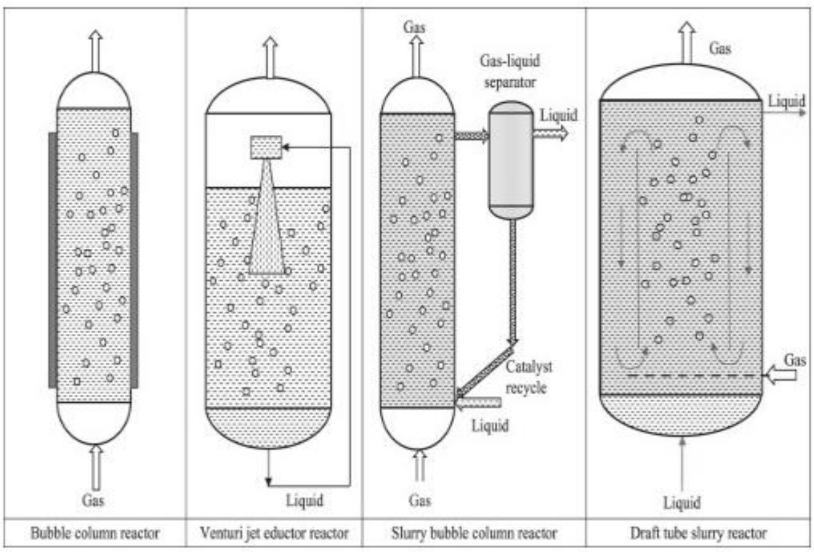


Figure -10. Variants of bubble column reactors.

Bubble columns offer several advantages, such as simple operation without any moving parts; excellent mixing, heat and mass transfer rates; low catalyst attrition rates; and the ability to accommodate bubble columns are backmixing in liquid phase (may result in lower a wide range of residence time requirements. Some of

the disadvantages of conversion and unfavorable selectivity) and limitations on catalyst size and loading.

5.4.6 TOWER FERMENTERS

Tower fermenters are mainly used t carry out continuous fermentation. This system was ist time used by Bass in 1870s which was 8.5m high and 1 m in diameter. This fermenter was developed to overcome the drawbacks faced by batch fermentation. It is mainly used in brewing industries for production of beer (Fernandez, 1996). A typical tower fermenter consists of a yeast gradient and a gradient of wort also going up the tower. The purpose of this multi stage fermenter is to provide flow of process with the help of gravity. Bulk of raw materials, water and malt are elevated to the top of fermenter first which then come downward without the need of any pump. An inlet is present at the bottom where as an outlet is present at the top. It also carries insulating jackets which are meant to maintain optimum temperature conditions for organisms to grow. Baffles are also present which are meant for agitation. Figure 11 shows the basic design of tower fermenter.

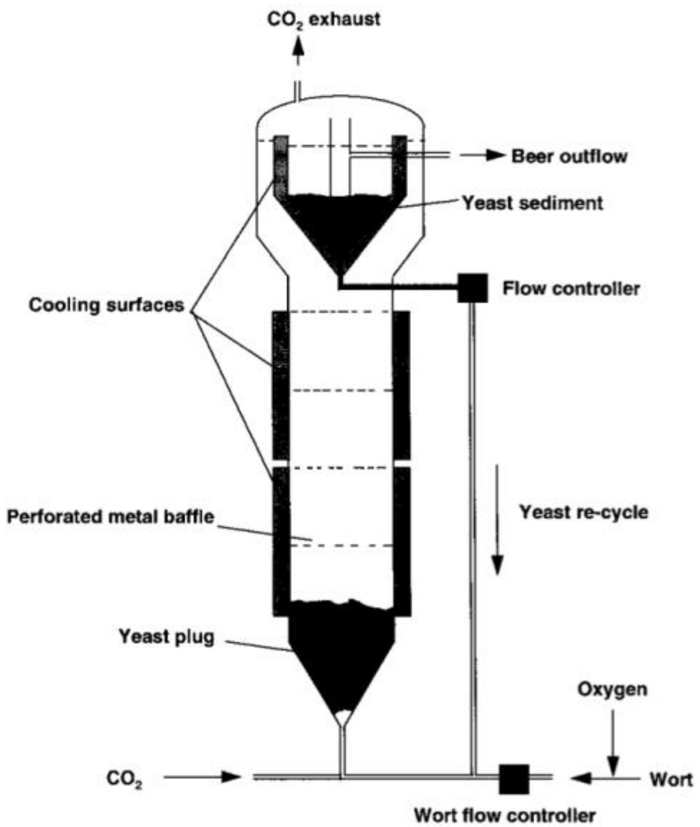


Figure -11. Basic design of a tower fermenter for yeast biomass production.

In a typical tower fermenter, there is a gradient of yeast and a gradient of wort gravity going up the tower (making the tower fermenter a heterogenous system). The tower relied on very flocculent yeast which formed a thick plug at the bottom of the tower. The very high concentrations of yeast at the bottom of the tower quickly consumed the available sugars in the wort. Green beer leaving a typical tower fermenter contained 0.5% yeast. The high concentration of yeast meant the beer fermented out very quickly 4 hours for anale or 8-12 for a lager. For comparison, Budweiser has a fermentation time of 100 hours plus or minus two hours.

5.4.7 BATCH BIOREACTORS

This type of bioreactor is widely used in processing industries. It is involved in variety of fermentation processes i.e. crystallization process, various chemical reactions, dissolution of solids, mixing of product, batch distillation, extraction of liquids and polymerization processes (Fig.1). It consists of a tank having and agitator and an incorporated heating and cooling system. They are of variable sizes ranging from less than a liter to higher than that of 15000 liters. Their fabrication is done using steel, glass lined steel, glass alloys etc. the internal solid and liquids are charged by using electric connections. Gases produced as a result of fermentation are discharged from the top whereas liquid product discharges from the bottom . Batch reactor is shown in figure 1. Advantages of the batch reactor are; it is advantageous because of its versatility, a serious of various operations can be carried out in a single vessel, it is useful in treating potent and toxicogenic compounds.

5.4.8 BIOREACTOR

These bioreactors can be applied to microbes involving processes like fermentation (alcoholic), acid (vinegar) production, waste water treatment etc. In this solute and solvents are added in proper amounts along with the enzymes (fig.12). For this purpose the enzymes are introduce using filters and pumps. These filter membranes are used for introduction of substrates and release of product. The membrane acts as a filter and don’t let the enzymes to leave the bioreactor, stirrer is used for the mixing. The materials used in the membrane are

cellulose acetate, polysulfonate and polyamide. Advantages of membrane bioreactor are as follows; there is very less enzyme loss due membrane presence. There is continuous addition of enzyme due to which enzyme lost during reaction is covered. The enzyme can be replaced easily by the substrate.

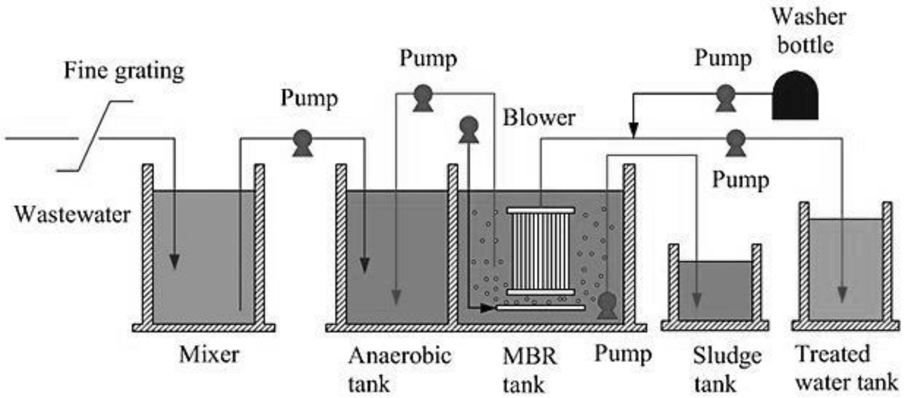


Figure 12: Membrane bioreactor.

5.4.9 PHOTO BIOREACTOR

These bioreactors are involved in fermentation processes which are to be carried out in the presence of light either sunlight or artificial light (Fig.13). This is a bioreactor that is used in the propagation of microorganisms utilizing light; these microbes are phototrophic in nature. These microbes have ability of photosynthesis like green plants and they can generate biomass using light. Because of the high expense by the use of artificial light, natural illumination i.e. sun is preferred. Important products produced by the use of photo bioreactors are asthaxantin ad p-carotene. Commonly glass or transparent plastic is used in their construction. They consist of an array of glass or tubes which are meant to capture light. Microbial culture is being circulated through these tubes and arrays by the use of airlift or centrifugation pumps. Cell sedimentation has to be avoided in this case which is done by the use of continuous cultures. Proper light penetration must be maintained and heating of tubes must be avoided by the use of cooling systems. The operation of photo bioreactors is continuous in nature and temperature is maintained at 35-40˚C. Fungi and cyanobacteria are used as microbial cultures, growing in sunlight and producing desired fermented products at night.



Figure -13. outdoor tubular photobioreactor.

Advantages of Photo bioreactor

Higher productivity rate can be achieved by using these bioreactors.They provide large surface to volume ratio to carry out fermentation process. Gas transfer can be controlled in a better way. Evaporation of growth media is reduced. The batch is protected from contamination. There is reduction of fouling due self-cleaning process of the tube. Algae are cultivated in a controlled way so its production is high. It is 10 to 20 times greater in this reactor than the bag reactors. Light usage is maximum in photo bioreactors which results in increased yield and productivity. Uniform temperature is provided.

5.5.10 CYLINDRO-CONICAL VESSELS

The use of cylindro-conical vessels in the brewing of lager was first proposed by Nathan (1930), but his ideas were not adopted for the brewing of lagers and beers until the 1960s. Breweries throughout the world have now adopted this method of brewing. The vessel (Fig. 14) consists of a stainless-steel vertical tube with a hemispherical top and a conical base with an included angle of approximately 70 degrees. Aspect ratios are usually 3:1 and fermenter heights are 10–20 m. Operating volumes are chosen to suit the individual brewery requirements, but are often 150,000– 200,000 dm^3. Vessels are not normally agitated unless a particularly flocculant yeast is used, but small impellers may be used to ensure homogeneity when filling with wort. In the vessel, the wort is pitched (inoculated) with yeast and the

NOTES

fermentation proceeds for 40–48 h. Mixing is achieved by the generation of carbon dioxide bubbles that rise rapidly in the vessel. Temperature control is monitored by probes positioned at suitable points within the vessel. A number of cooling jackets are fitted to the vessel wall to regulate and cause flocculation and settling of the yeast. The fermentation is terminated by the circulation of chilled water via the cooling jackets which results in yeast flocculation. Thus, it is necessary to select a yeast strain, which will flocculate readily in the period of chilling. Part of this yeast may be withdrawn and used for repitching another vessel. The partially cleared beer may be left to allow a secondary fermentation and conditioning. Some of the advantages of this vessel in brewing are:

1. Reduced process times may be achieved due to increased movement within the vessel.
2. Primary fermentation and conditioning may be carried out in the same vessel.
3. The sedimented yeast may be easily removed since yeast separation is good.
4. The maturing time may be reduced by gas washing with carbon dioxide.

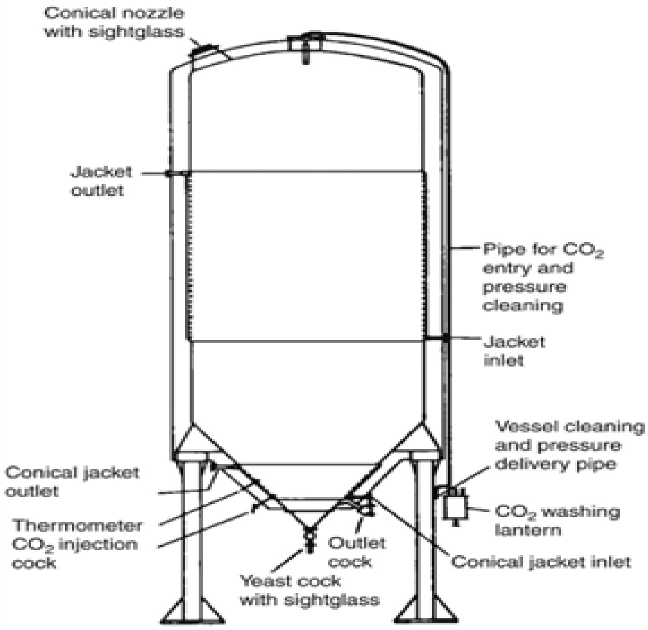


Figure 14: Cylindro - Conical Fermentation Vessel

5.5 ASEPTIC OPERATION AND CONTAINMENT

Aseptic operation involves protection against contamination and it is a well established and understood concept in the fermentation industries, whereas containment involves prevention of escape of viable cells from a fermenter or downstream equipment and is much more recent in origin. Containment guidelines were initiated during the 1970s. To establish the appropriate degree of containment which will be necessary to grow a microorganism, it, and in fact the entire process, must be carefully assessed for potential hazards that could occur should there be accidental release. Different assessment procedures have been used in the past depending on whether or not the organism contains foreign DNA [genetically engineered/modified (GM)]. Once the hazards are assessed, an organism can be classified into a hazard group for which there is an appropriate level of containment. The procedure, which was adopted within the European Community is outlined in Fig. 15. Nongenetically engineered organisms may be placed into a hazard group (1–4) using criteria to assess risk groups:

- The known pathogenicity of the microorganism.
- The virulence or level of pathogenicity of the microorganism—are the diseases it causes mild or serious?
- The number of organisms required to initiate an infection.
- The routes of infection.
- The known incidence of infection in the community and the existence locally of vectors and potential reserves.
- The amounts or volumes of organisms used in the fermentation process.
- The techniques or processes used.
- Ease of prophylaxis and treatment.

Once the organism has been allocated to a hazard group, the appropriate containment requirements can be applied (Table 4). Hazard group 1 organisms used on a large scale only require Good Industrial Large Scale Practice (GILSP). Processes in this category need to be operated aseptically but no containment steps are necessary, including prevention of escape of organisms. If the organism is placed in Hazard group 4 the stringent requirements of level 3 will have to be met before the process can be operated. Genetically engineered/modified organisms (GMOs) were classified as either harmless (Group I) or potentially harmful (Group II).

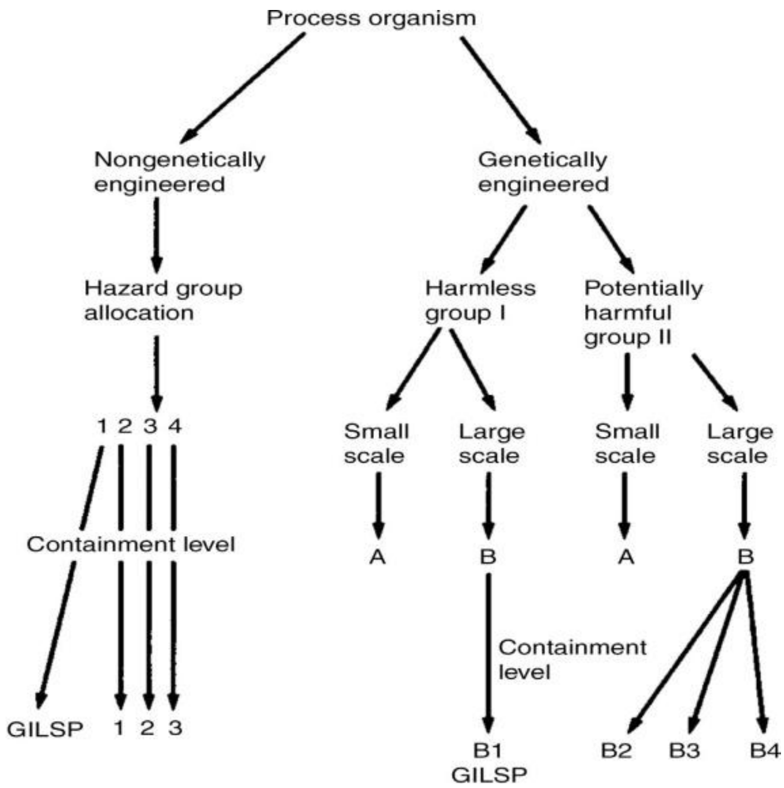


Figure 15: Categorization of a Process Microorganism and Designation of its Appropriate Level of Containment at Research or Industrial Sites Within the European Federation of Biotechnology

The process was then classified as either small scale (A: less than 10 dm^3) or large scale (B: more than 10 dm^3) according to the guidelines which can be found in the Health and Safety Executive, 1993. Therefore, large scale processes fell into two categories, IB or IIB. IB processes require containment level B1 and are subject to GILSP, whereas IIB processes were further assessed to determine the most suitable containment level, ranging from B2 to B4 as outlined in Table 4. Levels B2 to B4 correspond to levels 1 to 3 for non genetically engineered organisms.

In Oct. 2014, the UK's Health and Safety Executive issued the "The Genetically Modified Organisms (Contained Use) Regulations 2014," thus adopting EU regulations. The most important practical aspect of these regulations is that genetically modified and nongenetically modified organisms are considered under the same terms. Hence the A/B classification for small/large scale culture of GMOs has been removed. In Table 3 Summary of Safety Precautions

for Biotechnological Operations in the European Federation for Biotechnology (EFB).

Table-3: Summary of safety precautions for biotechnological operations (EFB)

Procedures	**GILSP**[a]	**Containment Category**		
		1	**2**	**3**
Written instructions and code of practice	+	+	+	+
Biosafety manual	–	+	+	+
Good occupational hygiene	+	+	+	+
Good Microbiological Techniques (GMT)	–	+	+	+
Biohazard sign	–	+	+	+
Restricted access	–	+	+	+
Accident reporting	+	+	+	+
Medical surveillance	–	+	+	+
Primary containment: Operation and equipment Work with viable microorganisms should take place in closed systems (CS), which minimize (m) or prevent (p) the release of cultivated microorganisms	–	m	P	P
Treatment of exhaust air or gas from CS	–	m	P	P
Sampling from CS	–	m	P	P
Addition of materials to CS, transfer of cultivated cells	–	m	P	P
Removal of material, products and effluents from CS	–	m	P	P
Penetration of CS by agitator shaft and measuring devices	–	m	P	P
Foam-out control	–	M	P	P
Secondary containment: Facilities				
Protective clothing appropriate to the risk category	+	+	+	+
Changing/washing facility	+	+	+	+
Disinfection facility	–	+		+
Emergency shower facility	–	–	+	+
Airlock and compulsory shower facilities	–	–	–	+
Effluents decontaminated	–	–	+	+
Controlled negative pressure	–	–	–	+
HEPA filters in air ducts	–	–	+	+
Tank for spilled fluids	–	–	–	+
Area hermetically sealable	–	–	–	+

m, Minimize release. The level of contamination of air, working surface and personnel shall not exceed the level found during microbiological work applying Good Microbiological Techniques. *p*, Prevent release. No

detectable contamination during work should be found in the air, working surfaces and personnel. [a]Unless required for product quality, –, not required; +, required.

In addition these regulations clarified their interaction with the 2002 COSHH (Control of Substances Hazardous to Health) regulations. The 2014 regulations (Schedule 1) describe the classes of contained use:

Class 1: Contained use of no or negligible risk. Containment level 1 is appropriate to protect human health and the environment.

Class 2: Contained use of low risk. Containment level 2 is appropriate to protect human health and the environment.

Class 3: Contained use of moderate risk. Containment level 3 is appropriate to protect human health and the environment.

Class 4: Contained use of high risk. Containment level 4 is appropriate to protect human health and the environment.

In 1989, the European Federation for Biotechnology were aware of this problem with nonrecombinant microorganisms and produced a consensus list. Most microorganisms used in large scale industrial processes are in the lowest hazard group which only require GILSP and GLP/GMP, although some organisms used in bacterial and viral vaccine production and other processes are categorized in higher groups. There is an obvious incentive for industry to use an organism, which poses a low risk as this minimizes regulatory restrictions and reduces the need for expensive equipment and associated containment facilities.

The fermenter is only one aspect of containment assessment. To meet the standards of the specific level of containment, it will also be necessary to consider the procedures to be used, staff training, the facilities in the laboratory and factory, downstream processing, effluent treatment, work practice, maintenance, etc. It will be necessary to ensure that all these aspects are of a sufficiently high standard to meet the levels of containment deemed necessary for a particular process by a government regulatory body. If these are met, then the process can be operated.

5.5.1 ACHIEVEMENT AND MAINTENANCE OF ASEPTIC CONDITIONS

Once the design problems of aeration and agitation have been solved, it is essential that the design meets the requirements of the degree of asepsis and containment demanded by the particular

process being considered. It will be necessary to be able to sterilize, and keep sterile, a fermenter and its contents throughout a complete growth cycle. There may be also a need to protect workers and the environment from exposure to hazardous microorganisms or animal cells. As mentioned earlier, the containment requirements depend on the size of the fermentation vessel.

The following operations may have to be performed according to certain specifications to achieve and maintain aseptic conditions and containment during a fermentation:

1. Sterilization of the fermenter.
2. Sterilization of the air supply and the exhaust gas.
3. Aeration and agitation.
4. The addition of inoculum, nutrients, and other supplements.
5. Sampling.
6. Foam control.
7. Monitoring and control of various parameters.

On a small scale, below 10 dm^3, the biohazard risk can be controlled by a combination of containment cabinets and work practices. When the volume of culture exceeds 10 dm^3, GILSP is required for those nonpathogenic nontoxigenic agents, which have an extended history of large scale use. For this category there should be prevention of contamination of the product, control of aerosols, and minimization of the release of microorganisms during sampling, addition of material, transfer of cells, and removal of materials, products, and effluents. It should be appreciated that the majority of fermentations fall into this category.

At higher containment levels, the following points need to be considered when designing a fermenter or other vessel, so that it can operate as a contained system:

1. All vessels containing live organisms should be suitable for steam sterilization and have sterile vent filters.
2. Exhaust gases from vessels should pass through sterile filters.
3. Seals on flange joints should be fitted with a single “O”-ring at the lower levels of containment. Flange joints on vessels for high hazard levels need double “O”-rings or double “O”-rings plus a steam barrier.
4. Appropriate seals should be provided for entry ports for sensor probes, inoculum, sampling, medium addition, acid, alkali, and antifoam.

5. Rotating shafts into a closed system should be sealed with a double acting mechanical seal with steam or condensate between the seals.
6. During operation a steam barrier should be maintained in all fixed piping leading to the "contained" vessels.
7. Provision of appropriate pressure relief facilities.

Check your Progress -1

Note: Write your answer in the space given below

a. Define HACCP.

b Uses of photobioreacor.

…………………………………………………………………………

5.6 LET US SUM UP

In this chapter, you have learned and gaining the knowledge of basic need of the fermenter, basic design of a fermenter, types of fermenter including photobioreacter, membrane bioreactor. Also, aseptic operation and containment of industrial operation has been leaned well through this chapter.

5.7 UNIT - END EXERCISES

1. Define HACCP and biosafety guidelines.
2. Discuss membrane bioreactor
3. Explain the continuous stirred tank bioreactor.

5.8 ANSWERS TO CHECK YOUR PROGRESS

1. Hazard Analysis of Critical Control Point (HACCP) it reals the aseptic operation and containment facilities of bioreactor.

2. Bioreactor used for culture and growth of algae, cyanobacteria nd any photosynthetic microorganism for fuel, pigments and Pharmacuticals.

5.14 SUGGESTED READINGS

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UNIT –VI: DESIGN OF A FERMENTOR - II

Structure

6.1 INTRODUCTION

A fermenter is an enclosed and sterile vessel that maintains optimal conditions for the growth of a microorganism. A fermentor is used for commercial production in fermentation industries and is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value. Fermentors are extensively used for food processing, fermentation, waste treatment, etcIn this chapter, construction of a fermenter starts with size, material selection for the vessel construction, baffles, spargers, and impellers of the fermentor are to be discussed.

6.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the body construction of a fermenter.
- Know the various parts of a fermenter
- Know the importance of baffles, spargers, and impellers in fermenter.
- Understand the concept of aeration and agitation.

6.3 BODY CONSTRUCTION

SIZE OF THE VESSEL

The size of fermentors ranges from 1-2 litre laboratory fementors to 5,00,000 litre or, occasionally, even more, fermentors of upto 1.2 million litres have been used. The size of the fermentor used depends

on the process and how it is operated. A summary of fermentor or size of fermentor (litres) Industrial product sizes for some common microbial fermentations is given in Table 1.

Table-1. Fermenter size vs. product.

Size of the fermenter (Lit)	**Product fermentation**
1 – 20,000	Diagnostic enzymes, substances for molecular research
40 – 80,000	Some industrial enzymes and antibiotics
100 – 1,50,000	Antibiotics such as penicillin, aminoglycosides, amylases, proteases, amino acids, steroids, wine , beer
2,00,000 – 5,00,000	Amino acids, wine, beer

In fermentations with strict aseptic requirements, it is important to select materials that can withstand repeated steam sterilization cycles. On a small scale (1–30 dm^3) it is possible to use glass and/or stainless steel. Glass is useful because it gives smooth surfaces, is nontoxic, corrosion proof, and it is usually easy to examine the interior of the vessel. Two basic types of fermenter are used:



Figure 1: Glass Fermenter With a Top-Flanged Carrying Plate (Inceltech L.H. Reading, England)

1. A glass vessel with a round or flat bottom and a top flanged carrying plate (Fig. 1). The large glass containers originally used were borosilicate battery jars. All vessels of this type have to be sterilized by autoclaving. Cowan and Thomas (1988) state that the largest practical diameter for glass fermenters is 60 cm.
2. A glass cylinder with stainless-steel top and bottom plates (Fig. 2). These fermenters may be sterilized in situ, but 30 cm diameter is the upper size limit to safely withstand working pressures. Vessels with two stainless steel plates cost approximately 50% more than those with just a top plate.

At pilot and industrial scale (Figs. 3 and 4), when all fermenters are sterilized in situ, any materials used will have to be assessed on their ability to withstand pressure sterilization and corrosion and on their potential toxicity and cost. Pilot-scale and industrial-scale vessels are normally constructed of stainless steel or at least have a stainless-steel cladding to limit corrosion. The American Iron and Steel Institute (AISI) states that steels containing less than 4% chromium are classified as steel alloys and those containing more than 4% are classified as stainless steels. Mild steel coated with glass or phenolic epoxy materials has occasionally been used. Wood, plastic, and concrete have been used when contamination was not a problem in a process. Galvanic corrosion may also be an issue when dissimilar metals are in direct contact or in contact via an electrolyte solution.



Figure 2 Three Glass Fermenters With Top and Bottom Plates (New Brunswick Scientific, Hatfield, England)

During the time of fermentation some extent the culture vessel may be corrodes, the extent posibility of vessel corrosion varied considerably and did not appear to be entirely predictable. Although stainless steel is often quoted as the only satisfactory material, it has been reported that mild-steel vessels were very satisfactory after 12-years use for penicillin fermentations and mild steel clad with stainless steel has been used for at least 25 years for acetone-butanol production. Pitting to a depth of 7 mm was found in a mild-steel fermenter after 7-years use for streptomycin production. The corrosion resistance of stainless steel is thought to depend on the

existence of a thin hydrous oxide film on the surface of the metal. The composition of this film varies with different steel alloys and different manufacturing process treatments such as rolling, pickling, or heat treatment. The film is stabilized by chromium and is considered to be continuous, nonporous, insoluble, and self-healing. If damaged, the film will repair itself when exposed to air or an oxidizing agent. The minimum amount of chromium needed to resist corrosion will depend on the corroding agent in a particular environment, such as acids, alkalis, gases, soil, salt, or fresh water. Increasing the chromium content enhances resistance to corrosion, but only grades of steel containing at least 10–13% chromium develop an effective film.



Figure 3 Stainless Steel Fully Automatic 10-dm^3 Fermenter Sterilizable-In-Situ (LSL, Luton, UK)

The inclusion of nickel in high percent chromium steels enhances their resistance and improves their engineering properties. The presence of molybdenum improves the resistance of stainless steels to solutions of halogen salts and pitting by chloride ions in brine or sea water. Corrosion resistance can also be improved by tungsten, silicone, and other elements.

AISI grade 316 steels, which contain 18% chromium, 10% nickel and 2–2.5% molybdenum are now commonly used in fermenter construction. In a citric acid fermentation where the pH may be 1–2 it will be necessary to use a stainless steel with 3–4% molybdenum (AISI grade 317) to prevent leaching of heavy metals from the steel which would interfere with the fermentation. AISI Grade 304, which contains 18.5% chromium and 10% nickel, is now used extensively for brewing equipment. The thickness of the

construction material will increase with scale. At 300,000– 400,000 dm^3 capacity, 7-mm plate may be used for the side of a vessel and 10-mm plate for the top and bottom, which should be hemispherical to withstand pressures.



Figure-4. Stainless steal pilot-plant.

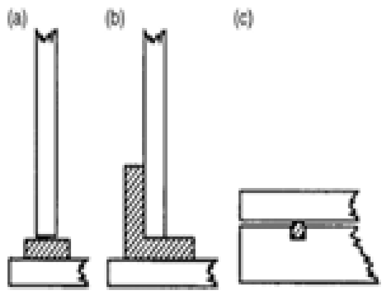


Figure-5 Joint seal. a) glass-glass; b) Glass-steal; c) Steal-steal.

At this stage, it is important to consider the ways in which a reliable aseptic seal is made between glass and glass, glass and metal, or metal and metal joints such as between a fermenter vessel and a detachable top or base plate. With glass and metal, a seal can be made with a compressible gasket, a lip seal or an "O" ring (Fig. 5). With metal to metal joints only an "O" ring is suitable. This is placed in a groove, machined in either of the end plate, the fermenter body or both. This seal ensures that a good liquid- and/or gas-tight joint is maintained in spite of the glass or metal expanding or contracting at different rates with changes in temperature during a sterilization cycle or an incubation cycle. Nitryl or butyl rubbers are normally used for these seals as they will withstand fermentation process conditions. These rubber seals have a finite life and should be checked regularly for damage or perishing.

A single "O" ring seal is adequate for lower containment levels, a double "O" ring seal is required for higher containment levels and a double "O" ring seal with steam between the seals (steam tracing) is necessary for more stringent containment.

Double seal without a steam trace has come advantages such as:

a. Double seals are more difficult to assemble correctly.
b. It is difficult to detect failure of one seal of a pair during operation or assembly.
c. Neither of the two seals can be tested independently.
d. Dead spaces between two seals must be considered for contamination.

6.4 TEMPERATURE CONTROL

Normally in the design and construction of a fermenter there must be adequate provision for temperature control, which will affect the design of the vessel body. Heat will be produced by microbial activity and mechanical agitation and if the heat generated by these two processes will not be ideal for the particular manufacturing process then heat may have to be added to, or removed from, the system. On a laboratory scale, little heat is normally generated and extra heat has to be provided by placing the fermenter in a thermostatically controlled bath, or by the use of internal heating coils or a heating jacket through which water is circulated.

Once a certain size has been exceeded, the surface area covered by the heating jacket becomes too small to remove the heat produced by the fermentation. When this situation occurs internal coils must be used and cold water is circulated to achieve the correct temperature. Different types of fermentation will influence the maximum size of vessel that can be used with jackets alone.

It is impossible to specify accurately the necessary cooling surface of a fermenter since the temperature of the cooling water, the sterilization process, the cultivation temperature, the type of microorganism, and the energy supplied by stirring can vary considerably in different processes. A cooling area of 50–70 m^2 may be taken as average for a 55,000 dm^3 fermenter and with a coolant temperature of 14°C the fermenter may be cooled from 120 to 30°C in 2.5–4 h without stirring. The consumption of cooling water in this size of vessel during a bacterial fermentation ranges from 500 to 2000 $dm^3\ h^{-1}$, while fungi might need 2000–10,000 $dm^3\ h^{-1}$, due to the

lower optimum temperature for growth. Such fermentations thus require water recycling with an inline chiller to minimize water usage.

To make an accurate estimate of heating/cooling requirements for a specific process it is important to consider the contributing factors. An overall energy balance for a fermenter during normal operation can be written as:

$$Q\text{met} + Q\text{ag} + Q\text{gas} = Q\text{acc} + Q\text{exch} + Q\text{evap} + Q\text{sen} \quad (1)$$

where Q_{met}, heat generation rate due to microbial metabolism; Q_{ag}, heat generation rate due to mechanical agitation; Q_{gas}, heat generation rate due to aeration power input; Q_{acc}, heat accumulation rate by the system; Q_{exch}, heat transfer rate to the surroundings and/or heat exchanger; Q_{evap}, heat loss rate by evaporation; Q_{sen}, rate of sensible enthalpy gain by the flow streams (exit—inlet).

Table 2 Representative Low and High Values of Calculated Heats (kcal dm^{-3} h^{-1}) of Fermentation for *Bacillus Subtilis* on Molasses at 37°C (Cooney et al., 1969)

	Heats of Fermentation					
	Q_{acc}	Q_{ag}	Q_{evap}	Q_{so}	Q_{exch}	Q_{met}
Low	3.81	3.32	0.023	0.005	0.61	1.12
High	11.3	3.31	0.045	0.010	0.65	8.65

This equation can be rearranged as:

$$Q\text{exch} = Q\text{met} + Q\text{ag} - Q\text{gas} - Q\text{acc} - Q\text{sen} - Q\text{evap} \quad (2)$$

Q_{exch} is the heat which will have to be removed by a cooling system.

Atkinson and Mavituna (1991b) have presented data to estimate Q_{met} for a range of substrates, methods to calculate power input for Q_{ag} and Q_{gas}, a formula to calculate the sensible heat loss for flow streams (Q_{sen}) and a method to calculate the heat loss due to evaporation (Q_{evap}). Based on the growth of *Bacillus subtilis* grown on molasses at 37°C (Table 2), the heat transfer rate can be calculated. They concluded that , Q_{evap} and Q_{sen} are small contributory factors and $Q_{\text{acc}} = 0$ in a steady-state system, Q_{evap} can also be eliminated by using a saturated air stream at the temperature of the broth.

When designing a large fermenter, the operating temperature and flow conditions will determine Q_{evap} and Q_{sen}, the choice of agitator, its speed, and the aeration rate will determine Q_{ag} and the sparger

design and aeration rate will determine Q_{gas}. The cooling requirements (jacket and/or pipes) to remove the excess heat from a fermenter may be determined by the following formula:

$$Q_{exch} = U \cdot A \cdot \Delta T \quad (3)$$

where A, the heat transfer surface available, m^2, Q, the heat transferred, W, U, the overall heat transfer coefficient, W/m^2K, ΔT, the temperature difference between the heating or cooling agent and the mass itself, K. The coefficient U represents the conductivity of the system and it depends on the vessel geometry, fluid properties, flow velocity, wall material, and thickness (Scragg, 1991). $1/U$ is the overall resistance to heat transfer (analogous to 1/K for gas-liquid transfer).

Atkinson and Mavituna (1991b) have given three methods to determine ΔT (the temperature driving force) depending on the operating circumstances. If one side of the wall is at a constant temperature, as is often the case in a stirred fermenter, and the coolant temperature rises in the direction of the coolant flow along a cooling coil, an arithmetic mean is appropriate:

$$\Delta T\text{am} = \frac{(T_f - T_e) + (T_f - T_i)}{2} \quad (4)$$

$$= \frac{T_f - (T_e - T_i)}{2} \quad (5)$$

where T_f, the bulk liquid temperature in the vessel; T_e, the temperature of the coolant entering the system; T_i, the temperature of the coolant leaving the system.

If the fluids are in counter- or cocurrent flow and the temperature varies in both fluids then a log mean temperature difference is appropriate:

$$\Delta T_m = \Delta T_e - \Delta T_i / \ln(\Delta T_e / \Delta T_i) \quad (6)$$

where ΔT_ε, the temperature of the coolant entering; ΔT_i, the temperature of the coolant leaving.

If the flow pattern is more complex than either of the two previous situations then the log mean temperature difference defined in Eq. (6) is multiplied by an appropriate dimensionless factor, which has been evaluated for a number of heat-exchanger systems by McAdams (1954).

Appropriate techniques have just been discussed to obtain values for Q_{exch}, U, and ΔT (or ΔT_{am} or ΔT_m).

$$A = \underline{Q_{exch}} \quad (7)$$

$U \, T\Delta$

Substituting in this equation makes it possible to calculate the heat-transfer surface necessary to obtain adequate temperature control.

6.5 AERATION AND AGITATION

The type of aeration-agitation system used in a particular fermenter depends on the characteristics of the fermentation process under consideration. Although fine bubble aerators without mechanical agitation have the advantage of lower equipment and power costs, agitation may be dispensed with only when aeration provides sufficient agitation, that is, in processes where broths of low viscosity and low total solids are used. Thus, mechanical agitation is usually required in fungal and actinomycete fermentations. Nonagitated (tower) fermentations are normally carried out in vessels of a height/diameter ratio of 5:1. In such vessels aeration is sufficient to produce high turbulence, but a tall column of liquid does require greater energy input in the production of the compressed air.

The structural components of the fermenter involved in aeration and agitation are:

a. The agitator (impeller).
b. Stirrer glands and bearings.
c. Baffles.
d. The aeration system (sparger).

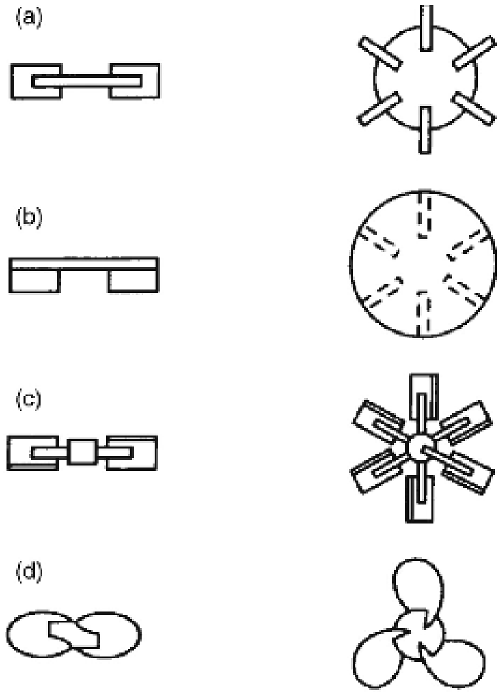
6.5.1 AGITATOR (IMPELLER)

The agitator is required to achieve a number of mixing objectives, for example, bulk fluid and gas-phase mixing, air dispersion, oxygen transfer, heat transfer, suspension of solid particles, and maintaining a uniform environment throughout the vessel contents. It should be possible to design a fermenter to achieve these conditions; this will require knowledge of the most appropriate agitator, air sparger, baffles, the best positions for nutrient feeds, acid or alkali for pH control, and antifoam addition. There will also be a need to specify agitator size and number, speed, and power input.

Agitators, depending on their type will impart either axial flow (parallel to the impeller shaft) or radial flow (perpendicular to the impeller shaft). Agitators may be classified as disc turbines, vaned discs, open turbines of variable pitch, and propellers together with more recent designs, and are illustrated in Figs. 6–7. The disc turbine (or Rushton turbine) is the most widely use fermenter agitator and

consists of a disc with a series of rectangular vanes set in a vertical plane around the circumference and the vaned disc has a series of rectangular vanes attached vertically to the underside. Air from the sparger hits the underside of the disc and is displaced toward the vanes where the air bubbles are broken up into smaller bubbles. The vanes of a variable pitch open turbine and the blades of a marine propeller are attached directly to a boss on the agitator shaft. In this case, the air bubbles do not initially hit any surface before dispersion by the vanes or blades.

Four other modern agitator developments, the Scaba 6SRGT, the Prochem Maxflo T, the Lightning A315, and the Ekato Intermig (Figs. 10 and 11), which are derived from open turbines, will also be discussed for energy conservation and use in high-viscosity broths.



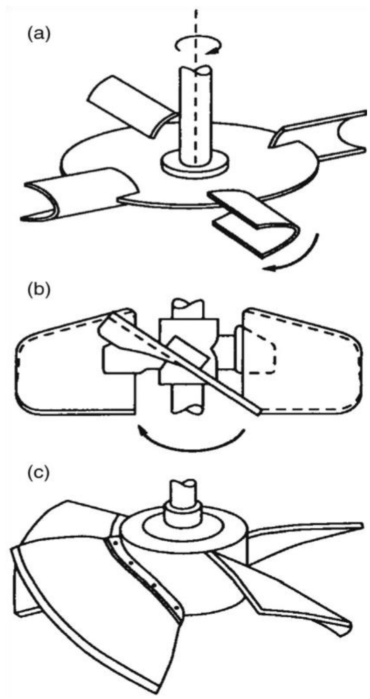


Figure 6: Types of agitators (a) Disc turbine; (b) vaned disc; (c) open turbine, variable pitch; (d) marine propeller (Solomons, 1969)

Figure 7: Diagrams of (a) Scaba agitator; (b) Lightnin' A315 agitator (four blades) and (c) Prochem Maxflo T agitator (four, five or six blades) (Nienow, 1990).

Rushton disc turbine of one-third the fermenter diameter has been considered the optimum design for use in many fermentation processes. It had been established experimentally that the disc turbine was most suitable in a fermenter since it could break up a fast air stream without itself becoming flooded in air bubbles. This flooding

condition is indicated when the bulk flow pattern in the vessel normally associated with the agitator design (radial with the Rushton turbine) is lost and replaced by a centrally flowing air-broth plume up the middle of the vessel with a liquid flow as an annulus. The propeller and the open turbine flood when V_s (superficial velocity, ie, volumetric flow rate/cross-sectional area of fermenter) exceeds 21 m h^{-1}, whereas the flat blade turbine can tolerate a V_s of up to 120 m h^{-1} before being flooded, when two sets are used on the same shaft. Besides being flooded at a lower V_s than the disc turbine, the propeller is also less efficient in breaking up a stream of air bubbles and the flow it produces is axial rather than radial. The disc turbine was thought to be essential for forcing the sparged air into the agitator tip zone where bubble break up would occur.

In other studies it has been shown that bubble break up occurs in the trailing vortices associated with all agitator types, which give rise to gas-filled cavities and provided the agitator speed is high enough, good gas dispersion will occur in low viscosity broths. It has been also shown that similar oxygen-transfer efficiencies are obtained at the same power input per unit volume, regardless of the agitator type.

In high-viscosity broths, gas dispersion also occurs from gas filled cavities trailing behind the rotating blades, but this is not sufficient to ensure satisfactory bulk blending of all the vessel contents. When the cavities are of maximum size, the impeller appears to be rotating in a pocket of gas from which little actual dispersion occurs into the rest of the vessel.

Relative dimensions are given as a proportion of the fermenter vessel diameter (T). Recently, a number of agitators have been developed to overcome problems associated with efficient bulk blending (mixing) and oxygen mass transfer in high viscosity/non-Newtonian fermentation broths. One approach is to combine two classes of impeller—one for mixing, the other for oxygen transfer. The second approach is to use a novel impeller type, which may also be used in combination.

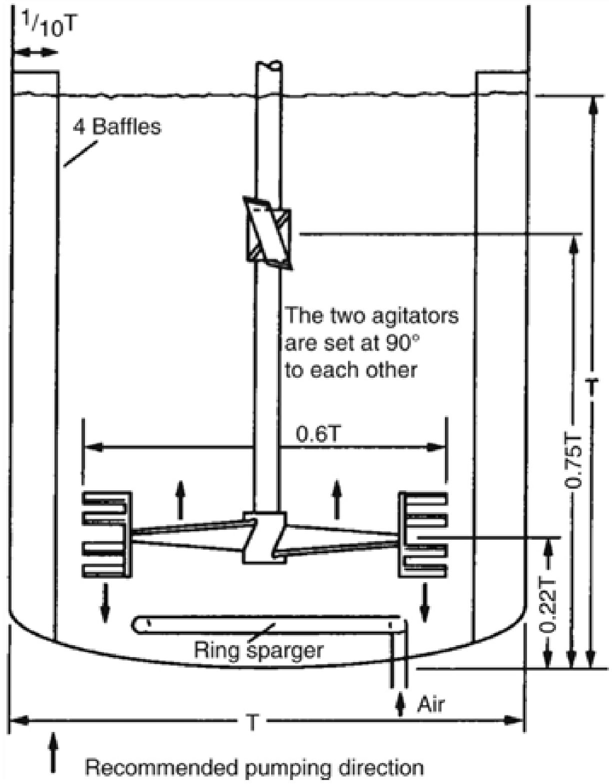


Figure 8 Arrangement for a Pair of Intermig Agitators (adapted from Stanbury).

The Scaba 6SRGT agitator is one, which at a given power input, can handle a high air flow rate before flooding. This radial-flow agitator is also better for bulk blending than a Rushton turbine, but does not give good top to bottom blending in a large fermenter which leads to lower concentrations of oxygen in broth away from the agitators and higher concentrations of nutrients, acid, or alkali or antifoam near to the feed points (Figs. 6 and 7). The top impeller, the Scaba impeller, installed to improve bulk flow (mixing), with the lower Rushton turbine used for gas dispersion. It has short mixing times, compared with other dual-impeller configurations without a draft tube. In addition mixing time was controlled by altering the speed of the Scaba impeller.

Another is the Prochem Maxflo agitator. It consists of four, five, or six hydrofoil blades set at a critical angle on a central hollow hub. A high hydrodynamic thrust is created during rotation, increasing the downward pumping capacity of the blades. This design minimizes the drag forces associated with rotation of the agitator such that the energy losses due to drag are low. This agitator is recommended for *Streptomyces* fermentation (800-dm^3 fermenter). The oxygen-transfer

efficiency was also significantly improved. It was thought that an improvement in bulk mixing was another contributing factor.

Intermitting agitators (Fig. 8) made by Ekato of Germany are more complex in design. Two units are used (with agitator diameter to vessel diameter ratios of 0.6–0.7) instead of a single Rushton turbine because their power number is so low. A large-diameter air sparger is used to optimize air dispersion. The loss in power is less than when aerating with a Rushton turbine. Air dispersion starts from the air cavities which form on the wing tips of the agitator blades. In spite of the downward pumping direction of the wings, the cavities extend horizontally from the back of the agitator blades, reducing the effectiveness of top to bottom mixing in a vessel.

Other modified version of Rushton turbines is BT-6 hydrofoil axial impellers, concluding that they are well suited for gas dispersion in fermenters where a broad range of gas flow rates is required. The improved version of the above is the Hayward Tyler B2 (formerly the APV-B2 or simply B2), an axial flow hydrofoil impeller, successfully retrofitted to a 550 dm^{-3} pilot- scale fermenter.

These new turbine designs make it possible to replace Rushton turbines by larger low power agitators, which do not lose as much power when aerated, are able to handle higher air volumes without flooding and give better bulk blending and heat/ mass transfer characteristics in more viscous media. However, there can be mechanical problems which are mostly of a vibrational nature.

Good mixing and aeration in high viscosity broths may also be achieved by a dual impeller combination, where the lower impeller acts as the gas disperser and the upper impeller acts primarily as a device for aiding circulation of vessel contents (a multirod mixing impeller). In a 15,000-dm^3 vessel, the same novobiocin yield and oxygen availability rate were obtained at about half of the power required by a standard turbine-stirred fermenter, but this type of impeller does not appear to have come into general use. Range of alternative triple-impeller configurations has improved aeration and agitation with low power consumption; e.g., improved glucoamylase production using *Aspergillus niger*.

Computational fluid dynamics (CFD) is a branch of fluid mechanics that uses computer based numerical analysis and algorithms to simulate, analyze and solve problems in fluid flow.

CFD was originally developed in the 1930s but it has seen rapid growth and application in more recent years due to the widespread availability of computers with increased computing power. The use of CFD in the analysis of mass transfer and mixing in a vessel fitted with triple novel NS impeller types (which had both up-pumping and down-pumping blades) in comparison with Rushton turbines. The key outcome of this analysis was that the novel impellers achieved higher levels of dissolved oxygen that the Rushton turbines under the same conditions. Gas-liquid mass transfer, bioreaction kinetics, and nonNewtonian behavior, are combined with CFD. The model developed can be used for both fermenter design and scale up.

6.5.2 BAFFLES

Baffles are flow-directing or obstructing vanes or panels used in some industrial process vessels, such as shell and tube heat exchangers, chemical reactors, and static mixers. Baffles are an integral part of the shell and tube heat exchanger design. Four baffles are normally incorporated into agitated vessels of all sizes to prevent a vortex and to improve aeration and agitation efficiency and to prevent vortexing. In vessels over 3-dm^3 six or eight baffles may be used. Baffles are metal strips roughly one-tenth of the vessel diameter and attached radically to the wall (Figs. 1 and 2 and Tables 2 and 3).

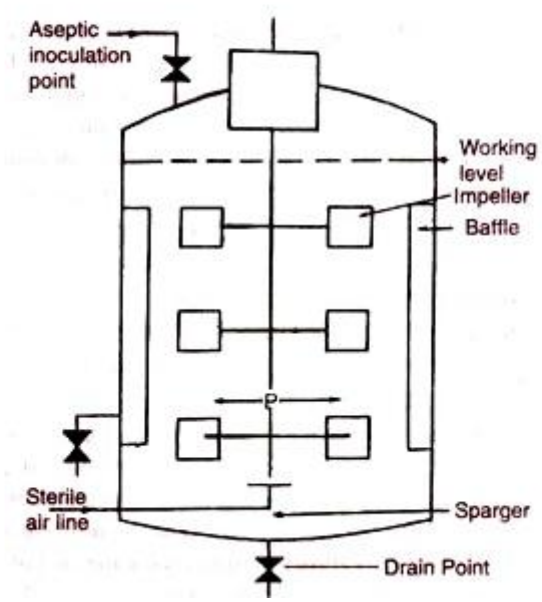


Figure-9: Basic fermenter designed with baffles.

The agitation effect is only slightly increased with wider baffles, but drops sharply with narrower baffles. Walker and

Holdsworth (1958) recommended that baffles should be installed so that a gap existed between them and the vessel wall, so that there was a scouring action around and behind the baffles, thus minimizing microbial growth on the baffles and the fermenter walls. Extra cooling coils may be attached to baffles to improve the cooling capacity of a fermenter without unduly affecting the geometry

6.5.3 AERATION SYSTEM [Sparger]

A sparger may be defined as a device for introducing air into the liquid in a fermenter. Three basic types of sparger have been used and may be described as the porous sparger, the orifice sparger (a perforated pipe), and the nozzle sparger (an open or partially closed pipe). A combined sparger-agitator may be used in laboratory fermenters (Fig. 10).

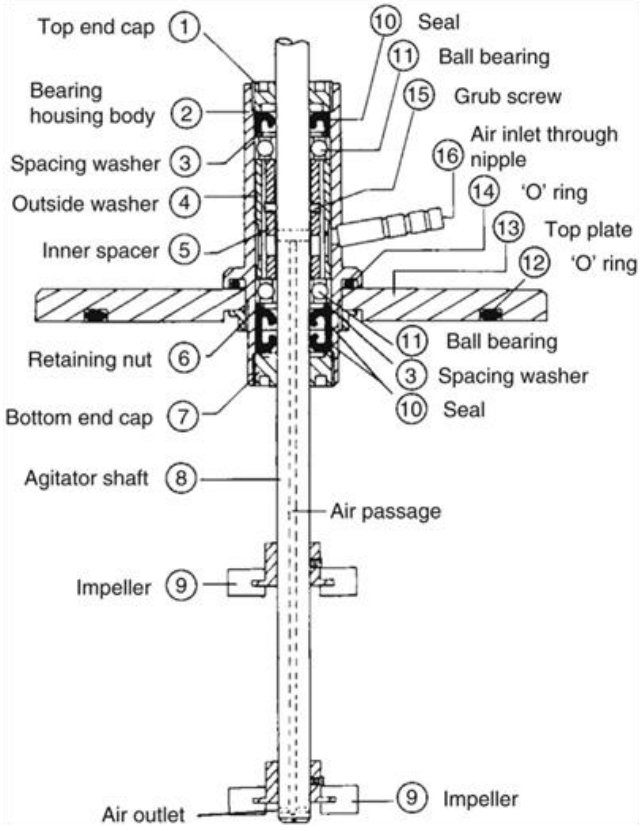


Figure 12 Diagram of Bearing Housing With Combined Agitator-Sparger (Inceltech L.H.)

Porous sparger: The porous sparger of sintered glass, ceramics or metal, has been used primarily on a laboratory scale in nonagitated vessels. The bubble size produced from such spargers is always 10–100 times larger than the pore size of the aerator block. The throughput of air is low because of the pressure drop across the sparger and there is also the problem of the fine holes becoming blocked by growth of the microbial culture.

Orifice sparger: Various arrangements of perforated pipes have been tried in different types of fermentation vessel with or without impellers. In small stirred fermenters, the perforated pipes were arranged below the impeller in the form of crosses or rings (ring sparger), approximately three-quarters of the impeller diameter. In most designs the air holes were drilled on the under surfaces of the tubes making up the ring or cross. Sparger holes should be at least 6 mm (1/4 inch) diameter because of the tendency of smaller holes to block and to minimize the pressure drop.

In low viscosity fermentations sparged at 1 vvm (volume of air^{-1} volume of medium^{-1} minute^{-1}) with a power input of 1 W kg^{-1}, the power often falls to below 50% of its unaerated value when using a single Rushton disc turbine which is one-third the diameter of the vessel and a ring sparger smaller than the diameter of the agitator. If the ring sparger was placed close to the disc turbine and its diameter was 1.2 times that of the disc turbine, a number of benefits could be obtained. A 50% higher aeration rate could be obtained before flooding occurred, the power drawn was 75% of the unaerated value, and a higher K_La could be obtained at the same agitator speed and aeration rate. These advantages were lost at viscosities of about 100 m Pas. Orifice spargers without agitation have been used to a limited extent in yeast manufacture, effluent treatment and later in the production of single-cell protein in the air-lift fermenter.

Nozzle sparger: Most modern mechanically stirred fermenter designs from laboratory to industrial scale have a single open or partially closed pipe as a sparger to provide the stream of air bubbles. Ideally the pipe should be positioned centrally below the impeller and as far away as possible from it to ensure that the impeller is not flooded by the air stream. The single-nozzle sparger causes a lower pressure loss than any other sparger and normally does not get blocked.

6.5.5 COMBINED SPARGER–AGITATOR

Herbert, Phipps, and Tempest (1965) developed the combined sparger-agitator design in small fermenter (1 dm^3), introducing the air via a hollow agitator shaft and emitting it through holes drilled in the disc between the blades and connected to the base of the main shaft. The design gives good aeration in a baffled vessel when the agitator is operated at a range of rpm (Fig. 12).

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6.6 STIRRER GLANDS AND BEARINGS

The satisfactory sealing of the stirrer shaft assembly has been one of the most difficult problems to overcome in the construction of fermentation equipment which can be operated aseptically for long periods. A number of different designs have been developed to obtain aseptic seals. The stirrer shaft can enter the vessel from the top, side or bottom of the vessel. Top entry is most commonly used, but bottom entry may be advantageous if more space is needed on the top plate for entry ports, and the shorter shaft permits higher stirrer speeds to be used by eliminating the problem of the shaft whipping at high speeds. Originally, bottom entry stirrers were considered undesirable as the bearings would be submerged.

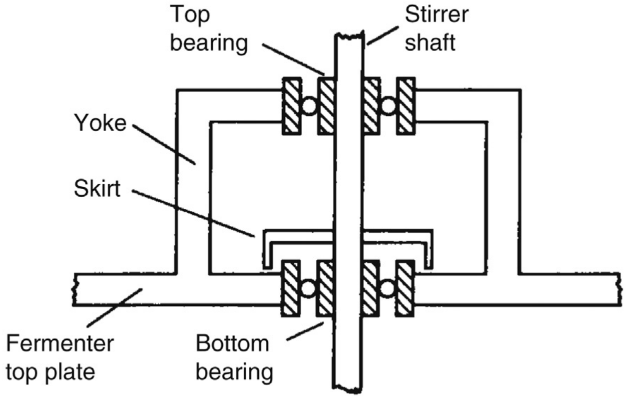


Figure **11.** A Simple Stirrer Seal Based on a Description Given by Rivett et al. (1950)

Mechanical seals can be used for bottom entry provided that they are routinely maintained and replaced at recommended intervals.

One of the earliest stirrer seals in a laboratory fermenter (Fig. 11) is a porous bronze bearing for a 13-mm shaft was fitted in the center of the fermenter top and another in a yoke directly above it. The bearings were pressed into steel housings, which screwed into position in the yoke and the fermenter top. The lower bearing and housing were covered with a skirt-like shield having a 6.5 mm overhang which rotated with the shaft and prevented airborne contaminants from settling on the bearing and working their way through it into the fermenter.

Four basic types of seal assembly have been used: the stuffing box (packed-gland seal), the simple bush seal, the mechanical seal, and the magnetic drive. Most modem fermenter stirrer mechanisms now incorporate mechanical seals instead of stuffing boxes and packed glands. Mechanical seals are more expensive, but are more durable and less likely to be an entry point for contaminants or a leakage point for organisms or products which should be contained. Magnetic drives, which are also quite expensive, have been used in animal cell culture vessels.

STUFFING BOX (PACKED-GLAND SEAL)

The stuffing box, the shaft is sealed by several layers of packing rings of asbestos (in the past) or cotton yarn, pressed against the shaft by a gland follower. At high stirrer speeds the packing wears quickly and excessive pressure may be needed to ensure tightness of fit. The packing may be difficult to sterilize properly because of unsatisfactory heat penetration and it is necessary to check and replace the packing rings regularly (Fig. 12). A modified versions are,

1. a split stuffing box with a lantern ring working in steam under pressure.
2. two stuffing boxes on the agitator shaft with a space in between kept filled with steam – used in large-scale vessels

These seals are sufficient for the requirements of lower containment requirements.

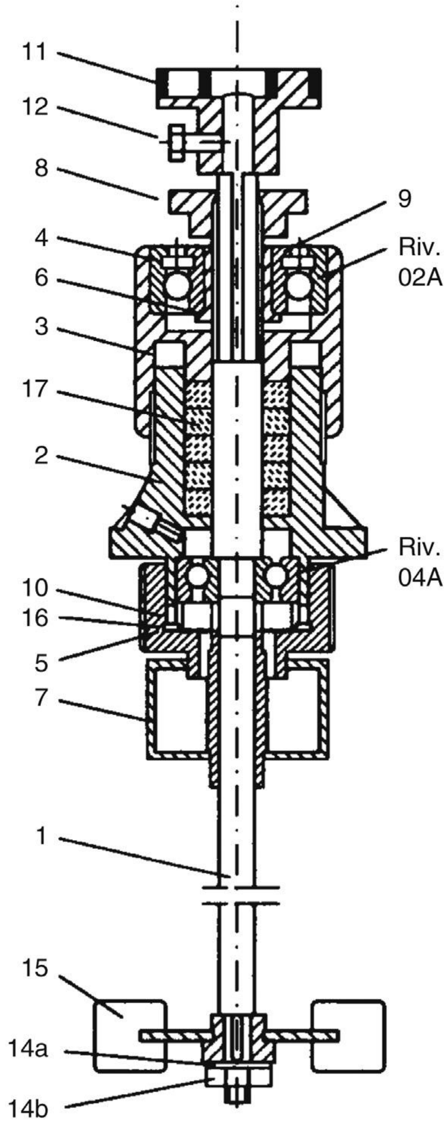


Figure 12. Packed-Gland Stirrer Seal (Chain et al., 1954). Components: *1*, agitator shaft; *2*, stuffing box; *3*, upper cap; *4*, lock ring; *5*, lower cap; *6*, chuck; *7*, greasecup; *8*, lock ring; *9*, lock nut; *10*, distance ring; *11*, half coupling; *12*, half coupling; *14a*, washer; *14b*, nut; *15*, impeller; *16*, shim; *17*, packing rings.

MECHANICAL SEAL

The mechanical seal assembly (Figs. 13 and 14) is now commonly used in both small and large fermenters. The seal is composed of two parts, one part is stationary in the bearing housing, the other rotates on the shaft, and the two components are pressed together by springs or expanding bellows. The two meeting surfaces have to be precision machined, the moving surface normally consists of a carbon-faced unit while the stationary unit is of stellite-faced stainless steel. Steam condensate can be used to lubricate the seals during operation and serve as a containment barrier. Single mechanical seals are used with

a steam barrier in fermenters for primary containment at lower levels, whereas double mechanical seals are typically used in vessels with the outer seal as a backup for the inner seal for primary containment at higher levels. With such higher hazards the condensate is piped to a kill tank. Monitoring of the steam condensate flowing out of the seal is an effective way for checking for seal failure. Disinfectants are alternatives for flushing the seals.

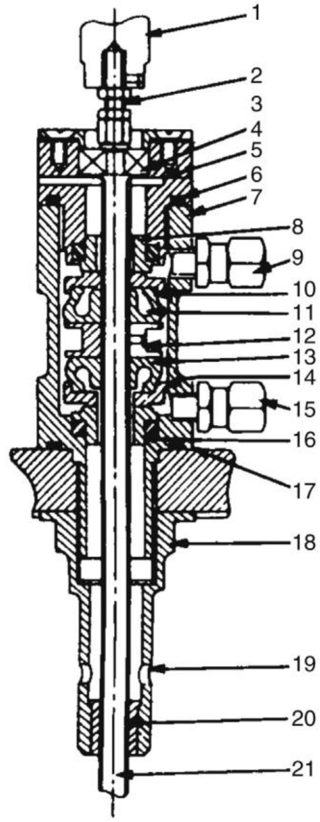


Figure 7.13 Mechanical Seal Assembly (Elsworth et al., 1958). Components: *1*, flexible coupling; *2*, stirrer shaft; *3*, bearing housing; *4*, ball journal fit on mating parts; *5*, two slots for gland leaks, only one shown; *6*, "O"-ring seal; *7*, seal body; *8*, stationary counter-face sealed to body with square-section gasket; *9*, exit port for condensate, fitted with unequal stud coupling; *10*, rotating counter-face; *11*, bellows; *12*, shaft muff; *13*, as 11; *14*, as 10; *15*, entry port for condensate, as 9; *16*, as 8; *17*, as 6; *18*, shaft bush support; *19*, leak holes; *20*, Ferobestos bush; *21*, ground shaft.

MAGNETIC DRIVES

The problems of providing a satisfactory seal when the impeller shaft passes through the top or bottom plate of the fermenter may be solved by the use of a magnetic drive in which the impeller shaft does not pierce the vessel. A magnetic drive (Fig.14) consists of two magnets: one driving and one driven. The driving magnet is held in bearings in a housing on the outside of the head plate and connected

to a drive shaft. The internal driven magnet is placed on one end of the impeller shaft and held in bearings in a suitable housing on the inner surface of the headplate. When multiple ceramic magnets have been used, it has been possible to transmit power across a gap of 16 mm. Using this drive, a Newtonian fluid can be stirred in baffled vessels of up to 300-dm^3 capacity at speeds of 300–2000 rpm. It

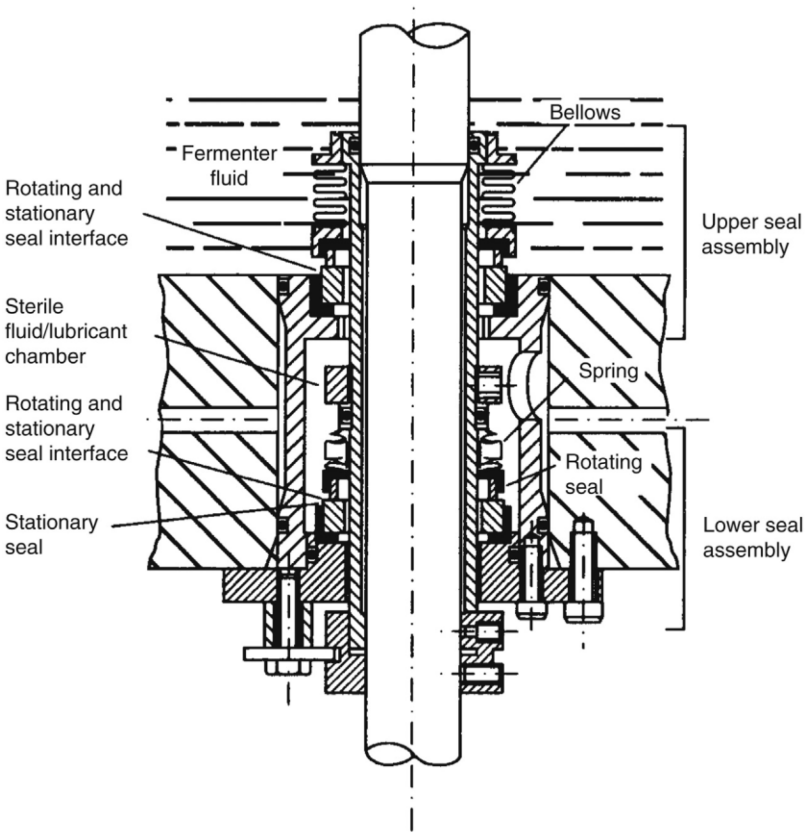


Figure.14 Double Mechanical Seal (New Brunswick Scientific, Hatfield, England)

Magnetic seal would be necessary to establish if adequate power could be transmitted between magnets to stir viscous mould broths or when wanting high oxygen transfer rates in bacterial cultures. It is suitable for microbial fermentations up to 1500 dm^3 which could be used when higher containment levels are specified. The stirring mechanism is ideal for animal cell culture to minimize the chances of potential contamination.

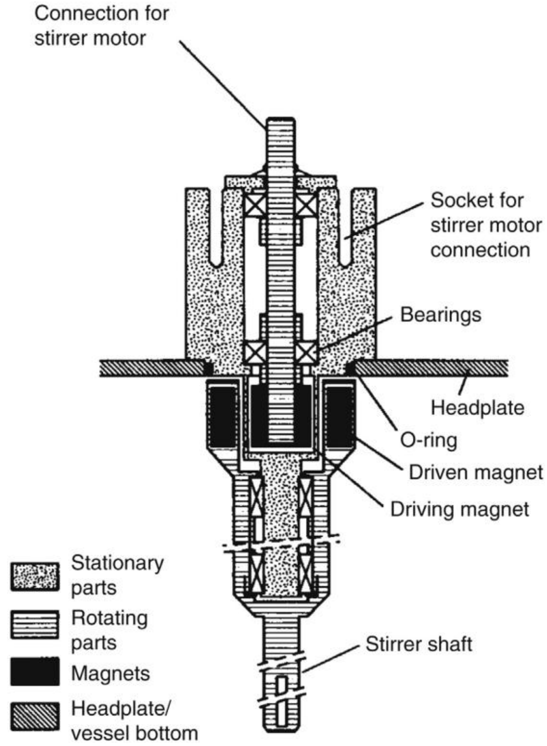


Figure.15. Magnetic seal.

6.7 VALVES

Valves attached to fermenters and ancillary equipment are used for controlling the flow of liquids and gases in a variety of ways. The valves may be:

1. Simple ON/OFF valves which are either fully open or fully closed.
2. Valves which provide coarse control of flow rates.
3. Valves which may be adjusted very precisely so that flow rates may be accurately controlled.
4. Safety valves which are constructed in such a way that liquids or gases will flow in only one direction (nonreturn valves).

When making the decision as to which valves to use in the design and construction of a fermenter, it is essential to consider the following points:

1. Valve should serve its chosen purpose, without any trouble.
2. The valve should withstand the rigors of the process. Hence the materials used to construct the valve should be suited to the process, and corrosive free.
3. Suitable fittings.

4. The valve can be operated automatically.
5. The cost and availability of suitable valves.
6. The valve should be used for containment purposes.

A wide range of valves is available, but not all of them are suitable for use in fermenter construction .

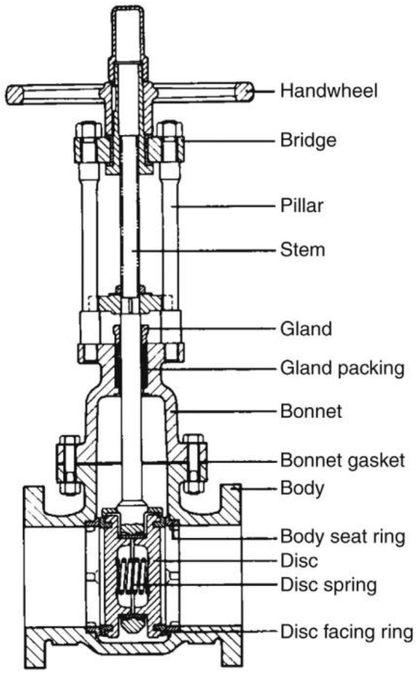


Figure 16 Sectional View of a Two-Piece Gate Valve (British Valve Manufacturers Association, 1972)

The valves described in this section open and close by (1) raising or lowering the blocking unit with a screw thread (rising stem), (2) a drilled sphere or plug, or a disc rotating in between two bearings, and (3) a rubber diaphragm or tube which is pinched.

1. GATE VALVES

In this valve (Fig. 17), a sliding disc is moved in or out of the flow path by turning the stem of the valve. It is suitable for general purposes on a steam or a water line for use when fully open or fully closed and therefore should not be used for regulating flow. The flow path is such that the pressure drop is minimal, but unfortunately it is not suitable for aseptic conditions as solids can pack in the groove where the gate slides, and there may be leakage round the stem of the valve which is sealed by a simple stuffing box. This means that the nut around the stem and the packing must be checked regularly.

2. GLOBE VALVES

In this valve (Fig. 18), a horizontal disc or plug is raised or lowered in its seating to control the rate of flow. This type of valve is very commonly used for regulating the flow of water or steam since it may be adjusted rapidly. It is not suitable for aseptic operation because of potential leakage round the valve stem, which is similar in design to that of the gate valve. There is a high-pressure drop across the valve because of the flow path.

In both the gate and globe valves it is possible to incorporate a flexible metallic membrane around the stem of the valve, to replace the standard packing. This modified type of valve can be operated aseptically, but is bigger and more expensive. Valves with nonrising stems have been used, but they are still potential sources of contamination.

3. PISTON VALVES

The piston valve (Fig. 19) is similar to a globe valve except that flow is controlled by a piston passing between two packing rings. This design has proved in practice to be very efficient under aseptic operation. It is important to sterilize them partly open so that steam can reach as far as possible into the valve body. There may be blockage problems with mycelial cultures. The pressure drop is similar to that of a globe valve.

4. NEEDLE VALVES

The needle valve (Fig. 20) is similar to the globe valve, except that the disc is replaced by a tapered plug or needle fitting into a tapered valve seat. The valve can be used to give fine control of steam or liquid flow. Accurate control of flow is possible because of the variable orifice formed between the tapered plug and the tapered seat. The aseptic applications are very limited.

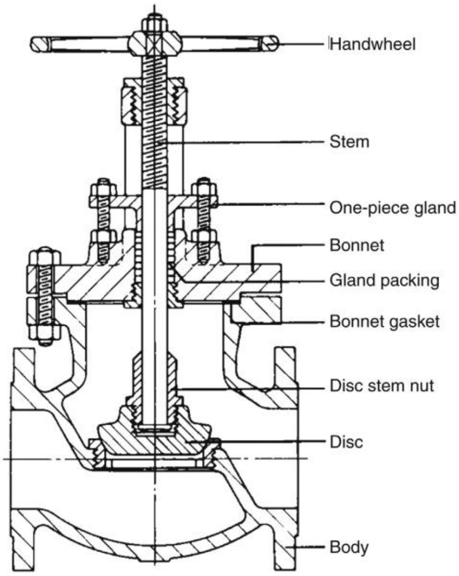


Figure 17 Globe Valve With Outside Screw and Conventional Disc (Kemplay, 1980).

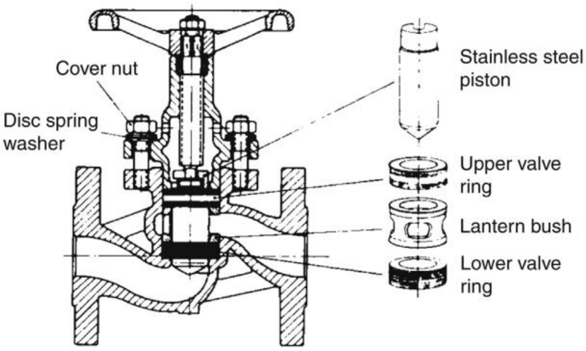


Figure 18 Piston Valve (Kemplay, 1980).

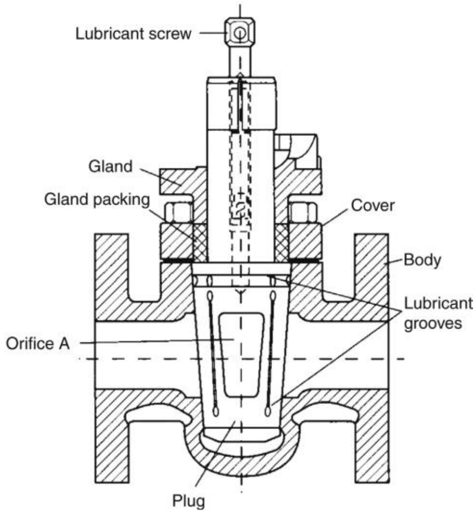


Figure 19 Sectional View of Lubricated Taper Plug Valve (British Valve Manufacturers Association, 1972).

5. PLUG VALVES

In this valve, there is a parallel or tapered plug sitting in a housing through which an orifice, A, has been machined. When the plug is turned through 90 degrees, the valve is fully open and the flow path is determined by the cross-sectional area of A, which may not be as large as that of the pipeline. This type of valve has a tendency to leak or seize up, but the use of lubricants and/or sealants may overcome these problems. If suitable packing sleeves are incorporated into the valve it will be suitable for use in a steam line as it is quick to operate, has protected seals, a minimal pressure drop, and a positive closure. It can also provide good flow control.

6. BALL VALVES

This valve (Fig. 20) has been developed from the plug valve. The valve element is a stainless-steel ball through which an orifice is machined. The ball is sealed between two wiping surfaces which wipe the surface and prevent deposition of matter at this point. The orifice in the ball can be of the same diameter as the pipeline, giving an excellent flow path. The valve is suitable for aseptic operation, can handle mycelial broths and can be operated under high temperatures and pressures. The pressure and temperature range is normally limited by the PTFE seat and stem seals.

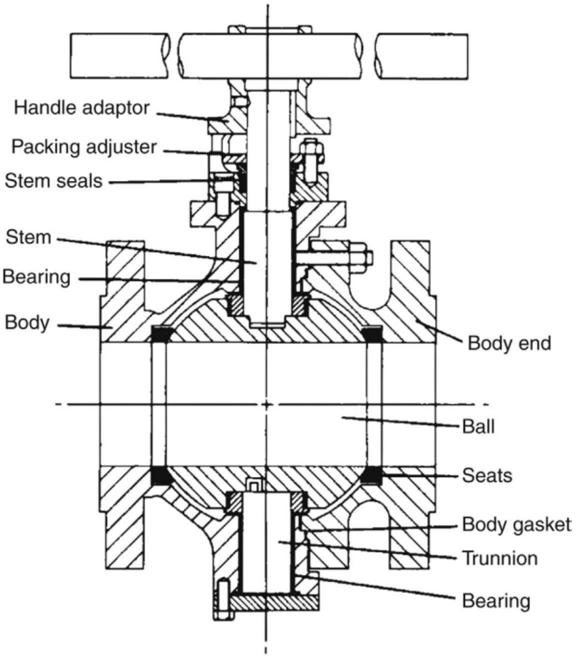
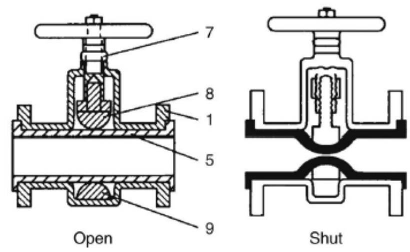


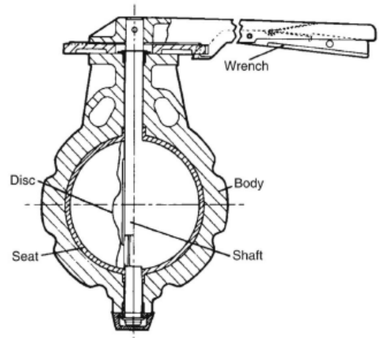
Figure 20 Sectional View of End-Entry Ball Valve (British Valve Manufacturers Association, 1972)

7. BUTTERFLY VALVES

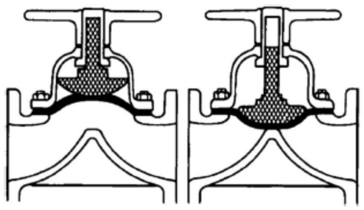
The butterfly valve (Fig. 21) consists of a disc which rotates about a shaft in a housing. The disc closes against a seal to stop the flow of liquid. This type of valve is normally used in large diameter pipes operating under low pressure where absolute closure is not essential. It is not suitable for aseptic operation.



Sectional View of Pinch Valve in Open and Shut Position
1, body; 5, flexible tube; 7, spindle; 8, top pinch bar; 9, lower pinch bar (Kemplay, 1980).



Sectional View of Wafer-Pattern Butterfly Valve



Sectional Views of Weir-Type Diaphragm Valves in Open and Closed Positions (Thielsch, 1967)

Figure – 21. Various valves

8. PINCH VALVES

In the pinch valve (Fig. 21), a flexible sleeve is closed by a pair of pinch bars or some other mechanism, which can be operated by compressed air remotely or automatically. The flow rate can be controlled from 10% to 95% of rated flow capacity. The valve is suitable for aseptic operation with fermentation broths, even when mycelial, as there are no dead spaces in the valve structure, and the closing mechanism is isolated from the contents of the piping. Obviously, the sleeve of rubber, neoprene, etc., must be checked regularly for signs of wear.

9. DIAPHRAGM VALVES

Like the pinch valve, the diaphragm valve (Fig.21) makes use of a flexible closure, with or without a weir. It may also be fitted with a quick action lever. This valve is very suitable for aseptic operation provided that the diaphragm is of a material which will withstand

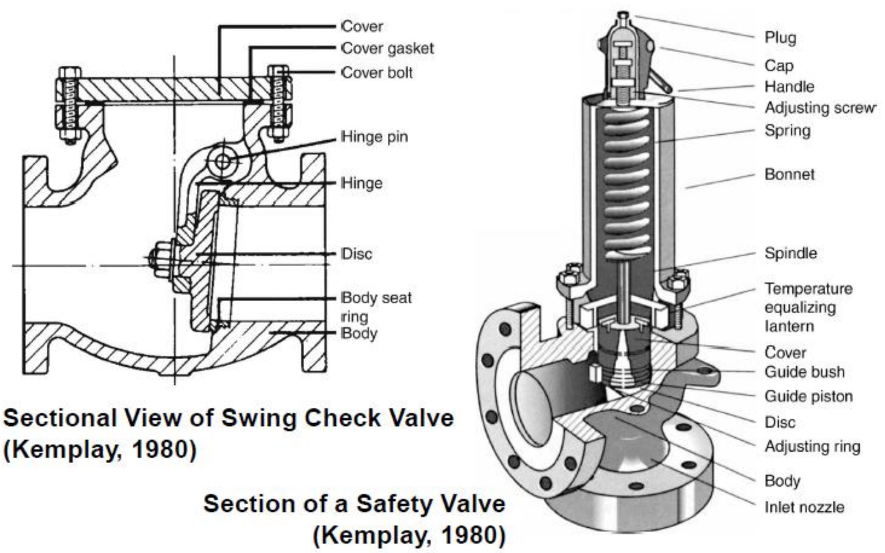
repeated sterilization. The valve can be used for ON/OFF, flow regulation, and for steam services within pressure limits. Diaphragm failure, which is often due to excessive handling, is the primary fault of the valve. Ethylene propylene diene modified (EPDM) is now the preferred material. Steam barriers on valves have been used by ICI plc for the “Pruteen” air-lift fermenter.

10. MOST SUITABLE VALVE

Among these group of valves which have just been described, globe and butterfly valves are most commonly used for ON/OFF applications, gate valves for crude flow control, needle valves for accurate flow control and ball, pinch or diaphragm valves for all sterile uses. Ball and diaphragm valves are now the most widely used designs in fermenter and other biotechnology equipment. Although ball valves are more robust, they contain crevices which make sterilization more difficult.

11. CHECK VALVES

The purpose of the check valve is to prevent accidental reversal of flow of liquid or gas in a pipe due to breakdown in some part of the equipment. There are three basic types of valve: swing check, lift check, and combined stop and check with a number of variants. The swing check valve (Fig. 22) is most commonly used in fermenter designs. The functional part is a hinged disc, which closes against a seat ring when the intended direction of flow is accidentally reversed.



Sectional View of Swing Check Valve (Kemplay, 1980)

Section of a Safety Valve (Kemplay, 1980)

Figure-22. Check valve & Safety valves.

12. PRESSURE-CONTROL VALVES

When planning the design of a plant for a specific process, the water, steam, and air should be at different, but specified pressures and flow rates in different parts of the equipment. For this reason, it is essential to control pressures precisely and this can be done using reduction or retaining valves.

13. PRESSURE-REDUCTION VALVES

Pressure-reduction valves are incorporated into pipelines when it is necessary to reduce from a higher to a lower pressure, and be able to maintain the lower pressure in the downstream side within defined limits irrespective of changes in the inlet pressure or changes in demand for gas, steam, or water.

14. PRESSURE-RETAINING VALVES

A pressure-retaining valve will maintain pressure in the pipeline upstream of itself, and the valve is designed to open with a rising upstream pressure. It is constructed with a reverse action of the pressure-reducing valve.

15. SAFETY VALVES

Safety valves must be incorporated into every air or steam line and vessel, which is subjected to pressure to ensure that the pressure will never exceed the safe upper limit recommended by the manufacturer or a code of practice and to satisfy government legislation and insurance companies. They must also be of the correct type and size to suit the operating conditions and be in sufficient numbers to protect the plant. The reliability of such valves is crucial.

In the simplest valves (Fig. 22), a spindle is lifted from its seating against the pressure of gas, steam or liquid. Once the pressure falls below the value set by the tensioned spring, the spindle should return to its original position. However, the valve may stick open if waste material lodges on the valve seat and plant operators may interfere with the release pressure setting. Bursting/rupture discs may be used as an alternative and are of a more hygienic design than some valves.

6.8 STEAM TRAPS

In all steam lines, it is essential to remove any steam condensate which accumulates in the piping to ensure optimum process

conditions. This may be achieved by incorporating steam traps, which will collect and remove automatically any condensate at appropriate points in steam lines. A steam trap has two elements. One is a valve and seat assembly which provides an opening, which may be of variable size, to ensure effective removal of any condensate. This opening may operate on an open/ close basis so that the average discharge rate matches the steam condensation rate or the dimensions of this opening may be varied continually to provide a continuous flow of condensate The second element is a device which will open or close the valve by measuring some parameter of the condensate reaching it to determine whether it should be discharged.

The steam trap may be designed to operate automatically on the basis of:

1. The density of the fluid by using a float (ball or bucket) which will float in water or sink in steam (Fig. 23).
2. By measuring the temperature of the fluid, closing the valve at or near steam temperature and opening it when the fluid has cooled to a temperature, say 8°C below the saturated steam temperature of the steam. The sensing element is often a stainless-steel capsule filled with a water-alcohol mixture, which expands when steam is present and presses a ball into a valve seat or contracts when cooler condensate is present and lifts the ball from the seating. These are used in thermostatic and balanced pressure steam traps (Figs. 23).

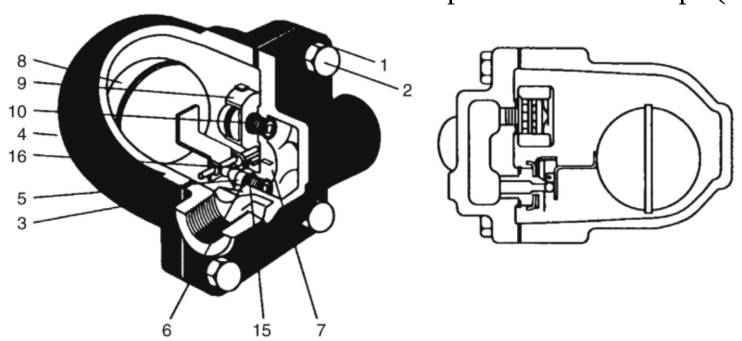


Figure 23 Ball Float Steam Trap With Thermostatic Air Vent (Spirax/Sarco, Cheltenham, England) *5*, Valve seat; *8*, ball float and lever; *9*, air vent.

3. By measuring the kinetic effects of the fluid in motion. At a given pressure drop, low-density steam will move at a much greater velocity than higher density condensate. The conversion of pressure energy into kinetic energy can be used to control the degree of opening of a valve. This type of steam trap is not used very widely.

4. Armer (1991) thinks that balanced pressure thermostatic traps are the most suitable for autoclaves and sterilizers. Some can operate close to steam-saturation temperature with little back up of condensate. Sarco Ltd (Cheltenham, UK) make a hermetically sealed balanced pressure steam trap (Fig. 7.42), which has been used with a high containment level pilot-plant fermenter (Hambleton et al., 1991).

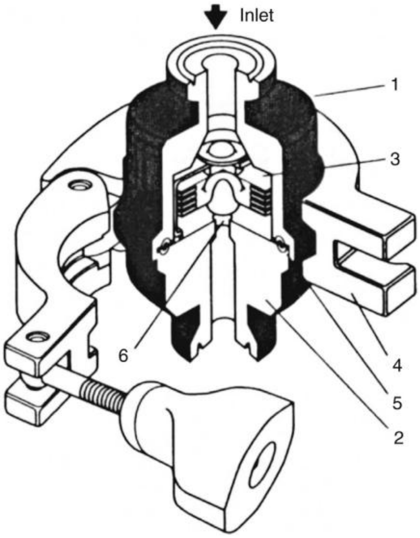


Figure 7.23 Balanced Pressure Steam Trap (Spirax/Sarco, Cheltenham, England) . *1*, Body; *2*, end connection; *3*, element; *4*, tri-clover clamp; *5*, Tri- Clover joint gasket; *6*, ball seat.

5. Any steam condensate may be (1) returned to the boiler, (2) used in a steam condensate line, (3) vented to waste, and (4) piped to a kill tank in a containment location.

Check your Progress -2

Note: Write your answer in the space given below

a. Name any tow safety valves.

b Functions of steam traps.

……………………………………………………..………………
… ……

6.9 LET US SUM UP

In this chapter, you have learned the basics of the fermenter vessel construction process. For the construction of an ideal fermenter, selection of material for constructing the vessel, other provisions such as stirrer glands and bearing, valves, safety valves, steam traps, baffles, spargers and impellers and the types and mechanism are clearly discussed. The understanding of the above information should

built you a suitable industrial manager to control the issues related with the fermenter operation.

6.10 UNIT - END EXERCISES

1. Discuss the various types of valves..
2. Describe the uses of impellers.
3. Explain the importance of body construction materials to design a industrial fermenter.

6.11 ANSWERS TO CHECK YOUR PROGRESS

1. HACCP – Hazard Ananlysis of Critical Control Point.
2. Algal cultivation.
3. Simple safety valve, Pressure control valve.
4. Remove steam condensate from the tubes. It may collect the condensate and remove automatically

6.14 SUGGESTED READINGS

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UNIT -VII: TYPES OF FERMENTATION

Structure

7.1 INTRODUCTION

Fermentation defined as "a chemical process by which molecules such as glucose are broken down anaerobically". More broadly, fermentation is the process of manufacture of wine and beer, a day-old process at least 10,000 years old. French chemist and microbiologist Louis Pasteur in the 19th century used the term fermentation in a narrow sense to describe the chemical changes brought about by yeasts and other microorganisms growing in the absence of air (anaerobically). Recently, various products are fermented through bioprocess industries, which may be fermented via either batch wise, or continuous process. In this chapter, types of fermentation such as batch, continuous, fed-batch methods and solid state fermentation and submerged fermentation have been discussed.

7.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the types of microbial fermentation.
- Know the process of batch, continuous and fed-batch system of industrial fermentation.
- Understand the principles and applications of solid state and submerged fermentations.

7.3 TYPES OF FERMENTATION

Fermentative microorganisms consume carbon sources, mainly sugar, to produce various acids, alcohols, and gases. In industry, fermentation is used to produce biopharmaceuticals, food and feed supplements, biofuels, and chemical building blocks. To establish a cost-effective process, bioprocess engineers have to consider various factors, including the costs for media and supplements, the process runtime, bacterial growth and viability, product titer and yield, and product quality. The concentrations of nutrients and by-products in the culture medium are important influencing factors. This is why, during process development, bioprocess engineers decide whether to apply a batch, fed-batch or continuous bioprocess

7.4 BATCH FERMENTATION

Batch fermentation is a closed culture system, because initial and limited amount of sterilized nutrient medium is introduced into the fermenter. Here, fermentation media is filled up to 80% space by fermentor, and the remaining space is used as head space. Head space plays an important role as some area of a fermentor is required for collection of air, gases, and foam which is produced during the fermentation process. The medium is inoculated with a suitable microorganism and incubated for a definite period for fermentation to proceed under optimal physiological conditions. Oxygen in the form of air, an antifoam agent and acid or base, to control the pH, are being added during the course of fermentation process (Fig. 1).

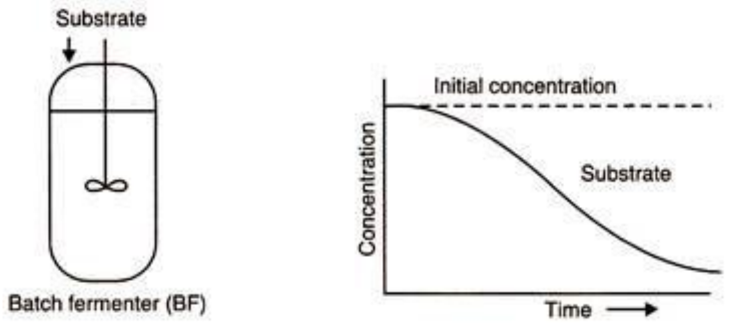


Figure-1: Batch fermenter.

During the course of incubation, the cells of the microorganism undergo multiplication and pass through different phases of growth (lag, log, stationary and decline) and metabolism due to which there will be change in the composition of culture medium, the biomass and

metabolites. The fermentation is run for a definite period or until the nutrients are exhausted. The culture broth is harvested and the product is separated. During the exponential growth phase, the microbes divide at a constant rate. When nutrients are getting depleted and by-products accumulate, growth slows down, and the culture enters the stationary growth phase. At this point, bioprocess engineers usually harvest the culture. If the culture continues, it would finally enter the death phase, which is characterized by a decrease in the viable cell density.

Batch fermentation may be used to produce biomass, primary metabolites and secondary metabolites under cultural conditions supporting the fastest growth rate and maximum growth would be used for biomass production. The exponential phase of growth should be prolonged to get optimum yield of primary metabolite, while it should be reduced to get optimum yield of secondary metabolites.

The used medium along with cells of microorganism and the product is drawn out from the fermenter. When the desired product is formed in optimum quantities, the product is separated from the microorganism and purified later on.

It has both advantages and disadvantages which are detailed below:

(i) Advantages

1. The possibility of contamination and mutation is very less.
2. Simplicity of operation and reduced risk of contamination.

(ii) Disadvantages

1. For every fermentation process, the fermenter and other equipment are to be cleaned and sterilized.
2. Only fraction of each batch fermentation cycle is productive.
3. It is useful in fermentation with high yield per unit substratum and cultures that can tolerate initial high substrate concentration.
4. It can be run in repeated mode with small portion of the previous batch left in the fermenter for inoculum.
5. Use of fermenter is increased by eliminating turn round time or down time.
6. Running costs are greater for preparing and maintaining stock cultures.
7. Increased, frequency of sterilization may also cause greater stress on instrumentation and probes.
8. Fresh sterilized medium and pure culture are to be made for every fermentation process.

9. Yield of the desired product may also vary.
10. There will be a non-productive period of shutdown between one batch productive fermentation to the other,
11. More personal (labors) are required.

Examples of batch fermentation includes, organic acid fermentation, amino acid fermentation, and certain secondary metabolites.

7.5 CONTINUOUS FERMENTATION

The majority of microbial fermentations can only be conducted over a short period of time in a batch or fed-batch culture, further increasing energy consumption and process complexity, and all these factors contribute to the high costs of the end products. Hence, an effort to make products more economically, increased awareness has been paid to developing open (unsterile) and continuous processes. If well conducted, continuous fermentation processes will lead to the reduced cost of industrial bio-products. To achieve cost-efficient open and continuous fermentations, the feeding of raw materials and the removal of products must be conducted in a continuous manner without the risk of contamination, even under 'open' conditions. In this method, fresh nutrient medium is added continuously or intermittently to the fermenter and equivalent amount of used medium with microorganisms is withdrawn continuously or intermittently for the recovery of cells or fermentation products (Fig. 2.).

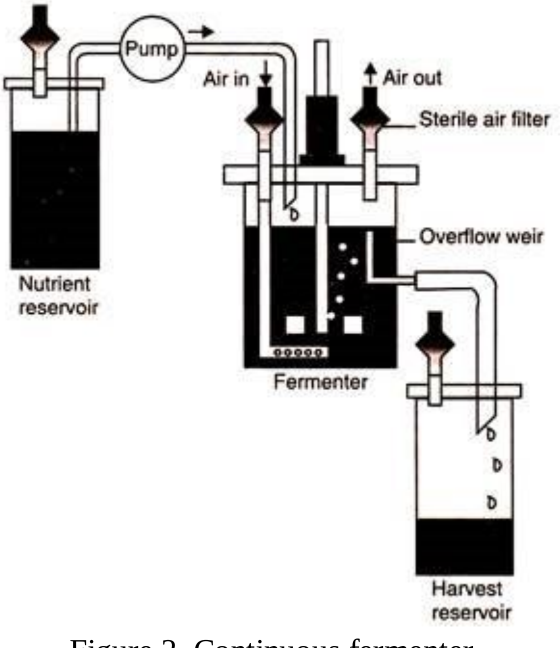


Figure 2. Continuous fermenter.

Microorganisms that can grow under extreme conditions such as high or low pH, high osmotic pressure, and high or low temperature, as well as under conditions of mixed culturing, cell immobilization, and solid state cultivation, are of interest for developing open and continuous fermentation processes.

As a result, volume of the medium and concentration of nutrients maintained at optimum level. This process has been operated in an automatic manner. The continuous fermenter has their maximum uses that take long time to reach high productivity, reduces down time and lowers the operating costs. In continuous mode, starting medium and inoculum are added to the fermenter. After the culture is grown the fermenter is fed with nutrients and broth is withdrawn at the same rate maintaining a constant volume of broth in the fermenter. In continuous mode with cell cycle, the cell mass is returned to the fermenter using micro filtrations with bacteria or screens with fungal mycelium.

A continuous fermentation is generally carried out in the following ways:

(a) Single stage fermentation
(b) Recycle fermentation
(c) Multiple stage fermentation

(a) Single Stage Fermentation: In this process, a single fermenter is inoculated and the nutrient medium and culture are kept in continuous operation by balancing the input and output of nutrient medium and harvested culture, respectively.

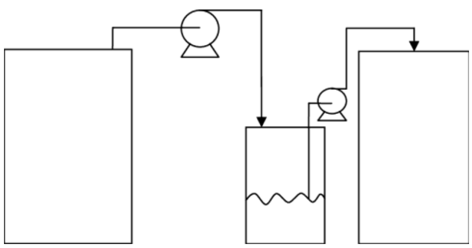


Figure-3 Simple continuous bioreactor**.**

(b) Recycle Fermentation: In this method, a portion of the medium is withdrawn and added to the culture vessel (return back). Thus, the culture is recycled to the fermentation vessel. This method is generally adopted in the hydrocarbon fermentation process. The recycling of cells provides a higher population of cells in the fermenter which results in greater productivity of the desired product.

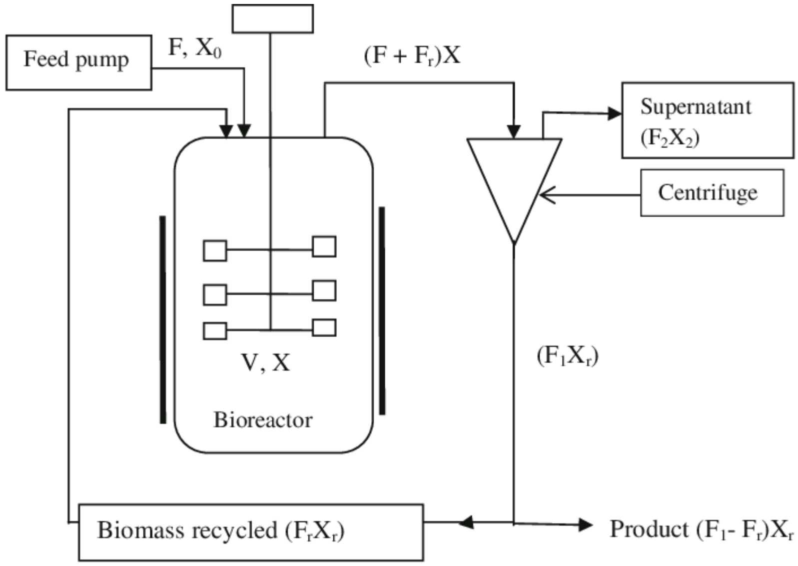


Figure. 4 Continuous fermentation with cells are recycled.

(c) Multiple Stage Fermentation: In this process, two or more fermenters are employed simultaneously and the fermentation is operated in a sequence. Different phases of fermentation process like growth phase and synthetic phase are carried out in different fermenters. Generally, growth phase is allowed in the first fermenter, synthetic phase in the second and subsequent fermenters. This process is adapted particularly to those fermentations in which growth and synthetic activities of the microorganisms are not simultaneous. Synthesis is not growth related but occurs when cell multiplication rate has slowed down.

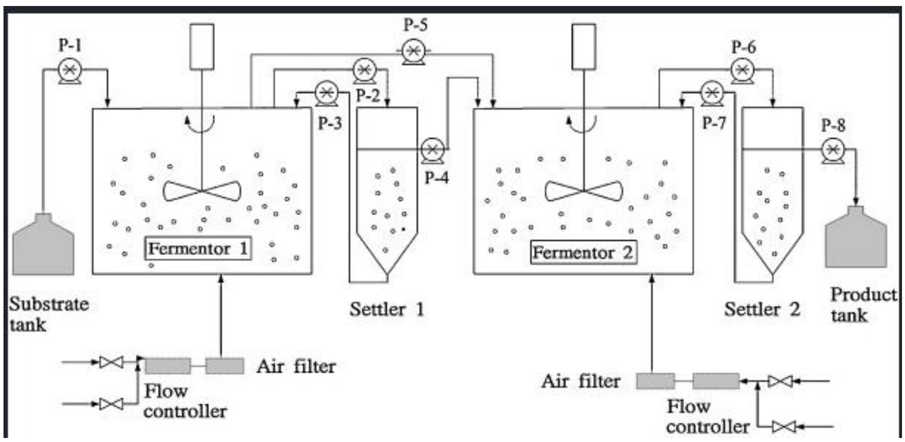


Figure-5: Multistage fermentation of ethanol production.

The process of continuous fermentation is monitored either by microbial growth activity or by product formation and these methods are called:

1. Turbidostat method, and
2. Chemostat method.

(i) Turbidostat Method: The first documentation of the turbidostat culture was by Bryson and Szybalski (1952). In this method the total cell content is kept constant by measuring the culture turbidity at a regular interval of fermentation process. By turbidity measurement it is possible to the fermenter to regulate both the nutrient feed rate and the culture withdrawal rate. Fermentation, in which this method is employed, must be carried out at a low maximum cell population which leads to the usage of less amount of substrate and wastage of greater amount of substrate as unused and residual medium, which is removed from the fermenter along with the harvested culture (Fig. 5).

All nutrients are still present in excess. The system works by a feedback loop to control the pump which supplies media into the vessel in response to the concentration of biomass detected in the culture. Media are added to the vessel when the biomass concentration is equal to or above the set point, and the media feed is stopped when the concentration of the culture decreases below the set point. In the turbidostat, the same basic growth equations as observed in the chemostat apply, but the setup does not allow the substrates to become limiting ($s_{residual} \gg K_s$). This is achieved by keeping the total biomass concentration in the vessel constant but at about 70–75% of the level of the maximum achievable biomass yield (g biomass/g carbon source used) for the growth medium. This is achieved by controlling the addition of fresh growth medium and ensuring that the working volume is constant.

If the concentration of the limiting substrate is much higher than its K_s ($s \gg K_s$), it follows that the term $[s/(K_s + s)]$ is very close to 1, and hence $\mu = \mu_{max}$. This means that a turbidostat can be operated without any nutrient limitations, but only at μ_{max} as opposed to a chemostat, where the growth rate can be set, but the culture is nutrient limited.

The advantage of applying the turbidostat approach is therefore that the growth of the culture is performed at the maximum specific growth rate of the microorganism because the nutrients are not limited in the media. The measured profile will therefore not be indicative of a particular nutrient limitation.

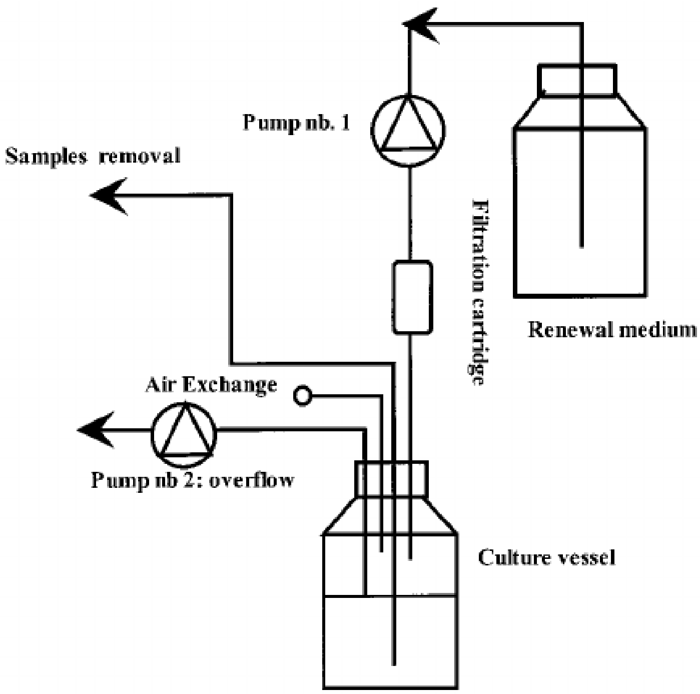


Figure-6: Turbidostat method of continuous fermentation.

(ii) Chemostat Method

The chemostat was originally introduced in the 1950s as a method to culture a bacterial population at a reduced growth rate for an indefinite period and is the most widely used approach to establish steady-state culture for various applications. In this method nutrient feed rate and harvest culture withdrawal rate are maintained at constant value. This is achieved by controlling the growth rate of the microorganism by adjusting the concentration of any one of the chemicals of the medium, like carbon source, nitrogen source, salts, O_2 etc. which acts as a growth limiting factor. Apart from the above chemicals, sometimes the concentration of the toxic product generated in the fermentation process, the pH values and even temperature also act as growth limiting factors. This method is employed more often than turbidostat method because of fewer mechanical problems and presence of less amount of unused medium in the harvested culture (Fig. 7).

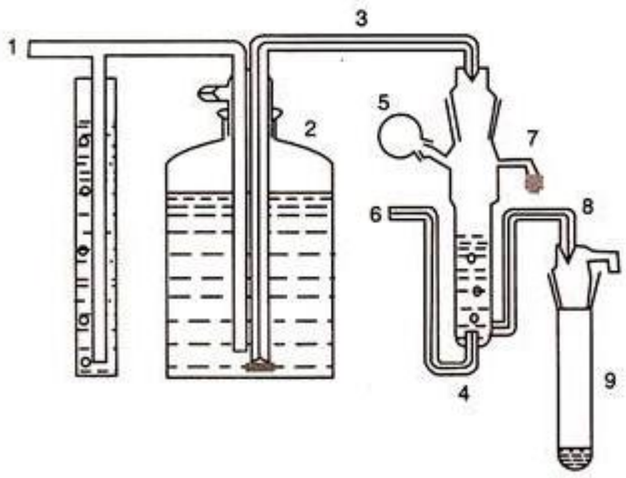


Figure-7: Chemostste operation. 1. air inlet; 2. Mariotte's bottle; 3. capillary for medium inlet; 4. fermenter; 5. inoculums point; 6. air inlet; 7. air outlet; 8. overflow capacity; 9. sampling tube**.**

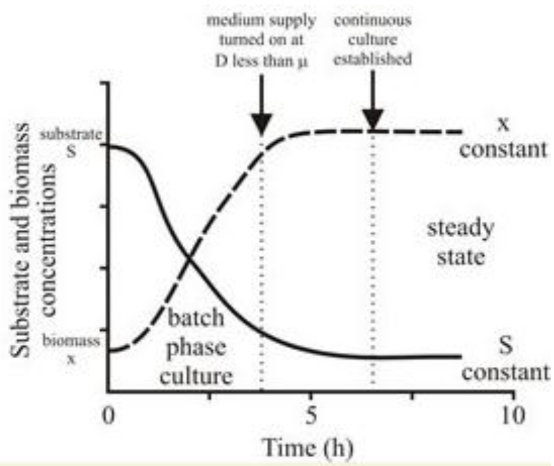


Figure-**8.** Record of biomass concentration (x) and substrate (glucose) concentration (S) over the early stages of a cultivation showing the transition from the initial batch phase to the continuous culture phase.

The initial studies provided the basis for the first theoretical description of continuous culture systems, which allowed the prediction of the steady-state concentrations of biomass and the residual concentration of the limiting substrate. In liquid medium, microbial growth is usually exponential and can be described by

$(1/x)(dX/dt)=d(\ln X)/dt=\mu=\ln 2/td$ ..... (1)

where *X* is the biomass expressed as dry weight per volume (g L^{-1}), *t* is the time, μ is the specific growth rate (h^{-1}), and t_d is the doubling time

(h). The μ and t_d can be assumed to be constant if all substrates necessary for growth are present in excess. Monod (1942) was the first to show that there is a correlation between the specific growth rate μ and the concentration of the limiting substrate according to

$$dX/dt=X\mu max[s/(Ks+s)] \text{ …… } (2)$$

where X is the biomass concentration at a given time, μ_{max} is the maximum specific growth rate (h^{-1}), s is the concentration of the limiting substrate, and K_s is the saturation constant and equals the substrate concentration at $0.5 \times \mu_{max}$. By converting Eq. (2) to $(dX/X)(1/dt) = \mu_{max}[s/(K_s + s)]$ and $(dX/X)(1/dt)$, being the increase of biomass over time or specific growth rate μ. Eq. (2) can be written as

$$\mu=\mu max[s/(Ks+s)] \text{ …… } (3)$$

In the chemostat, fresh nutrients are added to the culture at a fixed flow rate, the biomass and the products of metabolism are removed from the vessel at the same flow rate to maintain a fixed culture volume. The culture is maintained in a growth phase, where the doubling time and cell density are fixed and rigorous control of the environment is achieved. In the steady-state condition, the specific growth rate is equivalent to the dilution rate applied in the experiment and can only be operated at a growth rate below μ_{max}. If the dilution rate exceeds the maximum specific growth rate of the microorganism, the culture will become unstable and washout will occur. In a chemostat setup, the rate at which the growth-limiting substrate is supplied to the culture vessel is called the dilution rate (D) and thus equals the specific growth rate as described by Eqs. (4) and (5).

$$-dX/dt=DX \text{ ……… } (4)$$

Hence, an increase in biomass is equal to the growth minus the output.

$$dX/dt=\mu X-DX \text{ …….. } (5)$$

In the steady state, the biomass is constant and dX/dt is therefore zero and $\mu = D$. The dilution rate (D) is determined by the flow rate (F) expressed in mL h^{-1} of the feed pump and the culture's working volume (V) in mL, such that the hourly dilution rate is calculated by

$$D=FV \text{ …… } (6)$$

The constant medium supply and working volume results in a constant growth rate of the culture. The chemostat may be operated at different growth rates and limited by different growth-limiting nutrients in the media, which is generally selected to be carbon, nitrogen, sulfur, or phosphorus limitation.

However, continuous fermentations have certain advantages and limitations which are as follows:

(a) Advantages

1. The fermenter is continuously used with little or no shutdown time.
2. Only little quantity of initial inoculum is needed and there is no need of additional inoculum.
3. It facilitates maximum and continuous production of the desired product.
4. There is optimum utilization of even slow utilizable substances like hydrocarbons.

Continuous culture fermentation has been used for the production of single cell protein, antibiotics, organic solvents, starter cultures etc. (Table 1).

Table-1: Chemical Products produced in continuous fermentation

Growth associated	Non-growth associated
Acetic acid	Acetone
Butanediol	Butanol
Ethanol	Glycogen
Gluconic acid	Subtilin, nicin
Hydrogen sulfide	Chloramphenicol, Penicillin, Streptomycin
Lactic acid	Vitamin B12

(b) Disadvantages

1. Possibility of contamination and mutation because of prolonged incubation and continuous fermentation, are more.
2. Possibility of wastage of nutrient medium because of continuous withdrawal for product isolation.
3. The process becomes more complex and difficult to accomplish when the desired products are antibiotics rather than a microbial cells.
4. Lack of knowledge of dynamic aspects of growth and synthesis of product by microorganism used in fermentation.

Pilot plants or production plants have been installed for production of beer, fodder yeast, vinegar, baker's yeast. A wide variety of microorganisms are used for this type of fermentation (Table 2).

Table-2: Microbes used in continuous fermentation

Group of organism	Genera	Product
Actinobacteria	Streptomyces	Antibiotic
Algae	Cholerella, Scenedesmus	SCP, feed, pigments
Bacteria	Azotobacter, Rhizobium	Biofertilizer
	Bacillus	Enzymes and secondary metabolites
Fungi	Penicillium	Antibiotic
	Aspergillus	Citric acid, Enzymes
Yeast	Saccharomyces	Ethanol, SCP

Check your Progress-1

Note: Write your answer in the space given below

a. Name the commercial producer of citric acid.

b. Discuss the advantages of continuous fermentation.

…………………………………………………………………………

…………………………………………………………………………

7.6 FED-BATCH FERMENTATION

Fed-batch fermentation is a modified version of batch fermentation. It is the most common mode of operation in the bioprocess industry. In this process substrate is added periodically in instalments as the fermentation progresses, due to which the substratum is always at an optimal concentration. This is essential as some secondary metabolites are subjected to catabolite repression by high concentration of either glucose, or other carbohydrate or nitrogen compounds present in the medium. For this reason, the critical elements of the nutrient medium are added in low amount in the beginning of the fermentation and these substrates continue to be added in small doses during the production phase. This method is generally employed for the production of substances such as penicillin. Yoshida (1973) introduced this term for the first time for feeding the substrates to the medium as the nutrients are exhausted, so as to maintain the nutrients at an optimum level.

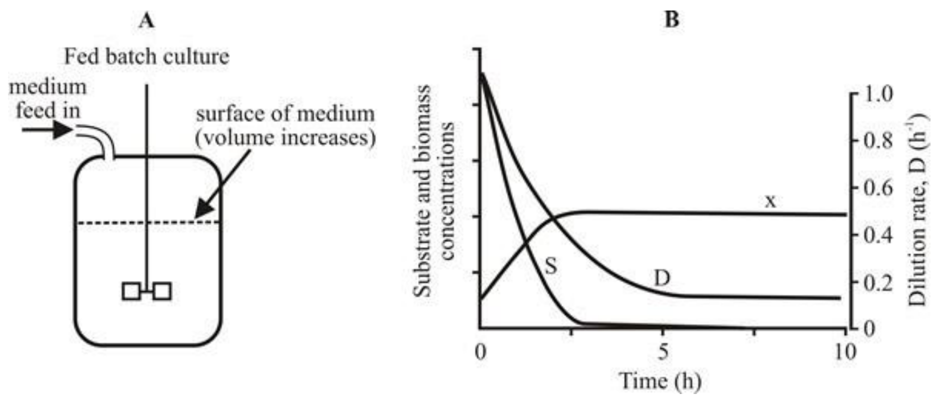


Figure-9. Fedbatch system of fermentation.

Although the total biomass in the culture increases with time, biomass concentration remains virtually constant because medium is added and the volume of the culture increases with time. Providing the dilution rate (D) is less than μ_{max} and K_s is much smaller than substrate concentration in the inflowing medium, a quasi-steady state may be achieved. However, unlike a continuous culture, D (and therefore μ) decreases with time, because the tank has a finite volume and as nothing is removed feeding with medium must stop at some stage in the cultivation (it was stopped at the 6 hour mark in the culture illustrated in Fig. 9).

The dilution rate of a fed-batch culture is given by:

$$D = \frac{F}{V_0 + F_t}$$

Where, D = dilution rate, V_0 = original volume of culture, and F = flow rate of medium into the culture.

The next development of the fed-batch culture is to remove some of the culture at intervals but to keep feeding the culture with more medium; this is a repeated fed-batch culture. In repeated fed-batch cultures the culture volume and consequently the dilution rate and specific growth rate undergo cyclical variations as parts of the culture are removed.

The fed-batch fermentation may be of three types:

1. **Variable Volume Fed Batch Culture:** The same medium is added resulting in an increase in volume.

2. **Fixed Volume Fed Batch Culture:** A very concentrated solution of the limiting substrate is added at a very little amount resulting in an insignificant increase in the volume of medium.
3. **Cyclic Fed Batch Culture:** As it is not possible to measure the substrate concentration by following direct methods during fermentation, which is necessary for controlling the feeding process, generally indirect methods are employed. For example – in the production of organic acids, the pH value may be used to determine the rate of glucose utilization.

Advantages

1. Production of high cell densities due to extension of working time (particularly growth associated products).
2. Controlled conditions in the provision of substrates during fermentation, particularly regarding the concentration of specific substrates for e.g. the carbon source.
3. Control over the production of, by products or catabolite repression, effects due to limited provision of substrates solely required for product formation.
4. The mode of operation can overcome and control deviations in the organism's growth pattern as found in batch fermentation.
5. Allows the replacement of water loss, by evaporation.
6. Alternative mode of operation for fermentations dealing with toxic substances or low solubility compounds.
7. Increase of antibiotic marked plasmid stability by producing the correspondent antibiotic during the time span of the fermentation.
8. No additional special piece of equipment is required as compared with the batch fermentation.
9. It is an effective method for the production of certain chemicals, which are produced at optimum level when the medium is exhausted like penicillin.

Disadvantages

1. It is not possible to measure the concentration of feeding substrate by following direct methods like chromatography.
2. It requires precious analysis of the microorganism. Its requirements and the understanding of its physiology with productivity is essential.
3. It requires a substantial amount of operator skill for the set-up of fermentation and development of the process.

4. In a cyclic fed batch culture, care should be taken in the design of the process to ensure that toxins do not accumulate to inhibitory levels and that nutrients other than those incorporated into the fed medium become limited also, if many cycles are run. The accumulation of non-producing or low producing variants may result.
5. The quantities of components to control must be above the detection limits of the available measuring equipment.
6. Fed-batch with recycle of cells can also be used for specific purpose such as ethanol fermentation and waste water treatment.

At present following products are being produced under fed batch culture:

1. Production of baker's yeast.
2. Penicillin production.
3. Production of Thiostrepton by Streptomyces laurentii
4. Production of industrial enzymes, histidine, glutathione (Brevibacterium flavum), Lysine (Corynebacterium glutamicum)

7.8 SUBMERGED FERMENTATION

Submerged fermentation is a method of manufacturing biomolecules in which enzymes and other reactive compounds are submerged in a liquid such as alcohol, oil or a nutrient broth. The process is used for a variety of purposes, mostly in industrial manufacturing.

Submerged production began in the 1930's and is the main method used today. Submerged liquid fermentations are traditionally used for the production of microbially derived enzymes. In the submerged process, the substrate used for fermentation is always in liquid state which contains the nutrients needed for growth.

The fermentor which contains the substrate is operated and the product biomass is harvested from the fermenter by using different techniques then the product is filtered or centrifuged and then dried. Submerged fermentation is a method of manufacturing biomolecules in which enzymes and other reactive compounds are submerged in a liquid such as alcohol, oil or a nutrient broth. The process is used for a variety of purposes, mostly in industrial manufacturing, and can be used to make products such as citric acid, glycerol or lactic acid.

Submerged culture fermentation has been widely used for the production of enzyme because in submerged fermentation unwanted metabolites are not produced and purification of enzymes takes place in an easy way. Submerged fermentation involves submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth. Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic meters. The fermentation media sterilizes nutrients based on renewable raw materials like maize, sugars, and soya. Most industrial enzymes are secreted by microorganisms into the fermentation medium in order to break down the carbon and nitrogen sources.

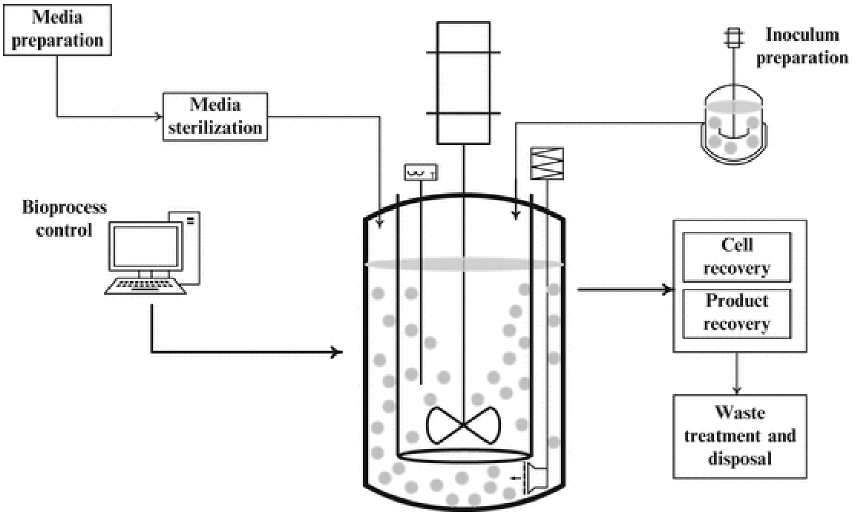


Figure – 10. Model of submerged fermentation. Agitator vessel.

Methods of submerged fermentation

Batch-fed and continuous fermentation processes are common. In the batch-fed process, sterilized nutrients are added to the fermenter during the growth of the biomass. In the continuous process, sterilized liquid nutrients are fed into the fermenter at the same flow rate as the fermentation broth leaving the system. Parameters like temperature, pH, oxygen consumption and carbon dioxide formation are measured and controlled to optimize the fermentation process. Next in harvesting enzymes from the fermentation medium one must remove insoluble products, e.g. microbial cells. This is normally done by centrifugation.

As most industrial enzymes are extracellular (secreted by cells into the external environment), they remain in the fermented broth after the biomass has been removed. The enzymes in the remaining broth are then concentrated by evaporation, membrane filtration or crystallization

depending on their intended application. If pure enzyme preparations are required, they are usually isolated by gel or ion exchange chromatography. Several types of submerged type of fermentors are known and they may be grouped in several ways: shape or configuration, whether aerated or anaerobic and whether they are batch or continuous. The most commonly used type of fermentor is the Aerated Stirred Tank Batch Fermentor.

Applications of submerged fermentation

- Submerged Fermentation/Liquid Fermentation utilizes free-flowing liquid substrates, such as molasses and broths.
- The bioactive compounds are secreted into the fermentation broth.
- The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients.
- This fermentation technique is best suited for microorganisms such as bacteria that require high moisture.
- An additional advantage of this technique is that the purification of products is easier.
- It is primarily used in the extraction of secondary metabolites that need to be used in liquid form

Table- 4: Major submerged fermentation products.

Type	**Products**
Organic acids	citric acid, lactic acid
Solvents	Alcohol, acetone-butanol, acetic acid
Secondary metabolites	Antibiotics, antiviral agents
Transformed products	Ergot alkaloids, steroid derivatives, and antitumor agent
SCP	Microbial biomass – spirulina, nostoc yeast, etc

Advantages of submerged fermentation

1. Measure of process parameters is easier than with solid-state fermentation.
2. Bacterial and yeast cells are evenly distributed throughout the medium.
3. There is a high water content which is ideal for bacteria.
4. Inoculum ration is usually small
5. Lower total investment costs

6. Improved process control
7. Reduced fermentation time
8. Reduced floor space requirements
9. Purification of products is easier
10. Lower labor costs
11. Simpler operations
12. Easier maintenance of aseptic conditions on an industrial scale.

Disadvantages

1. High costs due to the expensive media.
2. Expenses for equipment are higher
3. Consumption of electrical energy is higher
4. The process is very sensitive
5. Agitation is often essential
6. Chance of contamination

In recent years, many researchers have demonstrated that SSF has a large impact on productivity, leading to higher yields and improved product characteristics compared to SmF. However, it has Low volumetric productivity, relatively lower concentration of the products, more effluent generation and complex fermentation equipments, are the limitations of submerged fermentation.

7.9 SOLID-STATE FERMENTATION

In solid state fermentations, microbial growth occurs at the surface of solid substrates and the main difference from submerged liquid fermentation is the quantity of fluid involved. As described above, the biomass grows in suspension in the culture medium in liquid fermentations; whereas a solid state fermentation has a continuous gas phase between the particles or fragments of solid substrate, with a minimum of visible water.

The majority of the water in the solid state system is absorbed within the moist substrate particles and most of the space between particles is filled by the gas phase, although some water droplets may be visible between substrate particles and the particles themselves may be covered in a water film. This type of fermentation is one type of the more general solid substrate fermentation which can vary from processes that involve suspending the solid substrate particles in a continuous liquid phase (rather like food digestion in a stomach or rumen) through to processes (such as trickle filters) in which the liquid phase flows around and through an immobilised substrate.

Solid state fermentations can be divided into two groups according to the physical state of the substrate:

- Low moisture solids fermented with or without agitation, and
- Columns packed with solid substrate which is fermented as liquid is trickled through the column (with or without recirculation).

The substrate usually provides a rich and complex source of nutrients, which may not need to be supplemented. Mixed substrates of this sort are ideal for filamentous fungi able to form mycelia that can grow on and through particulate substrata producing a variety of extracellular enzymes. Traditional substrates for solid state fermentations are various agricultural products like cereal straw and other plant litter, and rice, wheat, maize, and soybean seeds. The majority of solid state fermentations involve filamentous fungi, though yeasts and bacteria can also be cultivated in this way; most are obligate aerobes (though there is no reason why the system should not be operated anaerobically) and the process may feature pure cultures of specific organisms or mixtures of several organisms.

Solid state fermenters are still the method of choice to produce spores of fungi for use as biological control agents. It is difficult to produce good fungal spores for biological control purposes in submerged culture (*Verticillium lecanii* is a notable exception). A major problem with solid state fermentations is that they are not easy to control and this can make it difficult to meet regulatory requirements. Composting processes can release noxious gases (ammonia and volatile sulphides in particular) and, in addition, aerial spores produced by the organisms involved may be potential health hazards as allergens.

Table-5: Selected solid state fermented products.

Products	**Substrate**	**Microorganism used**
Mushrooms	Straw manure	*Agaricus bisporus* *Lentinella edodes*
Cheese	Milk, curd	*Penicillium roquefotri*
Enzymes	Wheat bran, rice bran	*Aspergillus niger*
Organic acids	Can sugar, molasses	*Aspergillus niger*
Leaching of metals	Low grade ore	*Thiobacillus sp*
Sauekraut	Cabbage	Lactic acid bacteria
Composting	Organic waste	Fungi, bacteria, actinomycetes

Traditional examples of solid state fermentations are:

1. The formation of compost,
2. Mushroom cultivation (and, in a separate process, the production of starter cultures or mushroom spawn),
3. Leavening of bread dough, and
4. Mould ripening of cheeses, and other food products, such as salami and soy sauce.

What are effectively solid state fermentations also play a role in the production of chocolate and coffee. In these cases the fermentation is responsible for the preparation of the 'bean', to separate it from the fruit flesh and mucilage in cocoa; or the cherry and mucilage in coffee and impart flavour.

Cocoa products from the cacao tree, *Theobroma cacao*, are used in the food, chemical, pharmaceutical and cosmetic industries. The plant originated in the Amazon basin but is now an important cultivated crop worldwide in tropical regions. Cacao fruits are oval pods, 20 to 30 cm long and weighing in the region of 0.5 kg. They grow directly from the trunk and main branches of the tree and are usually harvested by hand. Each pod contains about 40 seeds (the cocoa 'beans', though they are not true beans) embedded in a thick pectinaceous pulp. Most of the initial processing is done on the farm. Harvested pods are cut open with a machete and the contents of pulp and cocoa seeds scooped out by hand. The rind is discarded, but pulp and seeds are piled in heaps for several days. During this time, the seeds and pulp undergo what is called 'sweating'; this is a natural microbial fermentation that liquefies the thick pulp so that it trickles away, leaving the cocoa seeds behind to be collected. Two simultaneous processes occur during their fermentation:

1. microbial activity in the mucilaginous pulp produces alcohols and organic acids as by-products of microbial metabolism, which also liberates heat, raising the temperature to about 50°C;
2. complex biochemical reactions occur within the seed cotyledons due to the diffusion of metabolites from the microorganisms and the rising temperature.

The fermentation features a succession of a range of yeasts, lactic acid bacteria, and acetic acid bacteria. Rapid growth of yeasts occurs in the first 24 hours (including *Saccharomyces cerevisiae*, *Kloeckera apiculata*, *Candida bombi*, *C. rugopelliculosa*, *C. pelliculosa*, *C. rugosa*, *Pichia fermentans*, *Torulospora pretoriensis*, *Lodderomyces*

elongiosporus, *Kluyveromyces marxianus* and *K. thermotolerans*). The most important roles of the yeasts are:

- breakdown of citric acid in the pulp leading to a change in pH from 3.5 to 4.2, which allows growth of bacteria,
- ethanol production (substrate for acetic acid bacteria),
- formation of organic acids (oxalic, succinic, malic and acetic),
- production of some organic volatiles which are probably precursors of chocolate flavour in the cocoa 'bean',
- secretion of pectinases to break down the pulp; pectin is the major plant polysaccharide responsible for the viscosity of the pulp and the yeast pectinases are needed to reduce viscosity of the pulp allowing fluid to drain away to increase aeration (required by acetic acid bacteria).

Unfermented cacao seeds do not produce chocolate flavour (their flavour is similar to that of raw potatoes) and it has not been possible to mimic the production of chocolate flavouring by treating fresh cacao seeds with hot alcohol and organic acids. Flavour development has an absolute requirement for the complex physical and organic biochemistry that occurs during the fermentation. The fermented beans are sun-dried before transfer to processing plants which roast the beans, then separate cocoa butter (used to make chocolate bars) from the solid matter (powdered to make cocoa for beverages and cooking.

Recently, the solid state approach has been developed for the commercial production of **extracellular enzymes** and other fungal products, and fungal spores for use as inoculum for biotransformations and, especially, as **mycopesticides**. The main advantage of this approach for microbial pesticide production is that the process can be done by individual farmers or local communes. Local production avoids some of the major problems associated with large scale commercial production facilities such as poor stability and short shelf-life, and associated storage and long distance shipping problems. The locally-produced mycopesticide can be much cheaper and the formulation can be optimised for local environmental conditions.

The fermenters used for large scale solid state fermentations are called **bioreactors**. They may be as simple as a plastic bag or an open tray of some sort, including even simple stacks of compost, which may be so large as to require heavy plant for mixing. More 'engineered' equipment might consist of stacked arrangements of trays through

which temperature and humidity controlled air is circulated, or rotary drum type bioreactors possibly including some additional agitation.

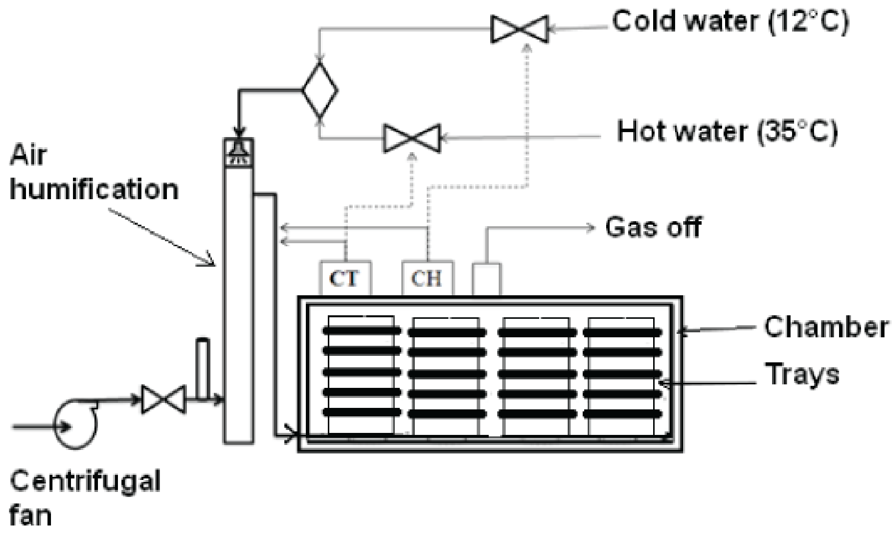


Figure-11. Solid state fermentation of enzyme lipase.

Solid state fermentation for enzymes

SSF has many advantages over SmF for microbial enzyme production namely high yield and productivities, lower operating costs, low-cost fermentation media (raw biomass, agro industrial residues), higher oxygen distribution, fewer operational troubles, simpler equipment and control system.

A comparative study on alkaline lipase production by a strain of *Aspergillus fumigatus* under SSF and SmF was made in which the maximum concentration of the enzyme was comparable in both cases. However, lipase produced by SSF was stable over a period of 15 days whereas lipase production in SmF decreased from day 5. Overall lipase produced by SSF to be 24% more active and 64% more thermally stable than lipase produced by SmF using a strain of *Rhizopus homothallicus.* These results confirm the interesting potential of SSF for producing lipase at low cost.

These are the advantages of solid-state fermentation:

1. The medium is cheap. Cereals, wheat bran, and other agricultural products can be used.
2. Special products can be produced. For example, red pigments, whose production is enhanced by solid-state fermentation.

3. The purification, recovery, and disposal of downstream processes in solid-state fermentation are usually simpler than in liquid fermentation.
4. Solid-state fermentation can produce food with a special flavor and improve nutritional value. For example, tempeh can be used as a substitute for meat, and its amino acid and fatty acids can be easily digested.
5. There is no wastewater discharge in solid-state fermentation.

However, there are some deficiencies in solid-state fermentation. Solid-state fermentation is a near-natural state fermentation, and there are many differences between it and submerged fermentation (SmF), in which the most significant feature is the low water activity and nonuniformity. Cell growth, and secretion of absorbed nutrients and metabolites are not uniform throughout, so that the detection and control of the fermentation parameters are more difficult. A number of biosensors used in liquid fermentation cannot be applied to solid-state fermentation. It is difficult to achieve large-scale industrial production of solid-state fermentation. The main reasons include easy contamination, low substrate utilization rate, the lack of solid-state fermentation reactor design, and so on.

Check your Progress -2
Note: Write your answer in the space given below
c. Give the applications of SSF.
d Name any four metabolites produced via SMF
………………………………………………………………………………

7.10 LET US SUM UP

In this chapter, you have learned and gaining the knowledge of types of microbial fermentation, more broadly, types of fermentation such as batch, continuous, fed-batch methods and solid state fermentation and submerged fermentation have been discussed. the principle, applications and the advantages and disadvantages are deeply discussed

7.11 UNIT - END EXERCISES

1. What are the types of continuous fermentation?
2. Discuss about beyond batch fermentation.
3. Explain the solid state fermentation of industrial enzymes.

7.12 ANSWERS TO CHECK YOUR PROGRESS

1. *Corynebacterium glutamicum*.
2. The fermenter is continuously used with little or no shutdown time. Only little quantity of initial inoculum is needed and there is no need of additional inoculum. It facilitates maximum and continuous production of the desired product. There is optimum utilization of even slow utilizable substances like hydrocarbons
3. The medium is cheap. Cereals, wheat bran, and other agricultural products can be used. Special products can be produced. For example, red pigments, whose production is enhanced by solid-state fermentation. The purification, recovery, and disposal of downstream processes in solid-state fermentation are usually simpler than in liquid fermentation.
4. Ethanol, Antibiotics, Enzymes, Acetic acid.

7.14 SUGGESTED READINGS

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UNIT -VIII: AEROBIC AND ANAEROBIC FERMENTATION

Structure

8.1 INTRODUCTION

Fermentation is usually defined as alcoholic fermentation by yeast. Yeast can ferment the sugar to alcohol in anaerobic environment, however many microbial metabolites are fermented through the microorganisms require certain amount of oxygen, known as aerobic fermentation. In this chapter, both aerobic fermentation and anaerobic fermentation methods and its merits and their limitations are discussed. In addition, dual and multiple fermentation process is also be gien in this chapter.

8.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the mechanism of fermentation.
- Know the process of aerobic and anaerobic fermentation process.
- Understand the principles and applications duel and multiple fermentations.

8.3 AEROBIC FERMENTATION

Aerobic fermentation is a metabolic process by which cells metabolize sugars and carbohydrates through fermentation in the presence of oxygen and occurs through the repression of normal respiratory metabolism (the crabtree effect in yeast). This process is fairly rare and

is primarily observed in yeasts. Aerobic fermentation is identified in different microorganisms including *Saccharomyces*, *Dekkera*, *Schizosaccharomyces*. Crabtree-positive yeasts will respire when grown with very low concentrations of glucose or when grown on most other carbohydrate sources. The Crabtree effect is a regulatory system whereby respiration is repressed by fermentation, except in low sugar conditions. When *Saccharomyces cerevisiae* is grown in low sugar concentration, and undergoes a respiration metabolism, the fermentation pathway is still fully expressed, while the respiration pathway is only expressed relative to the sugar availability.

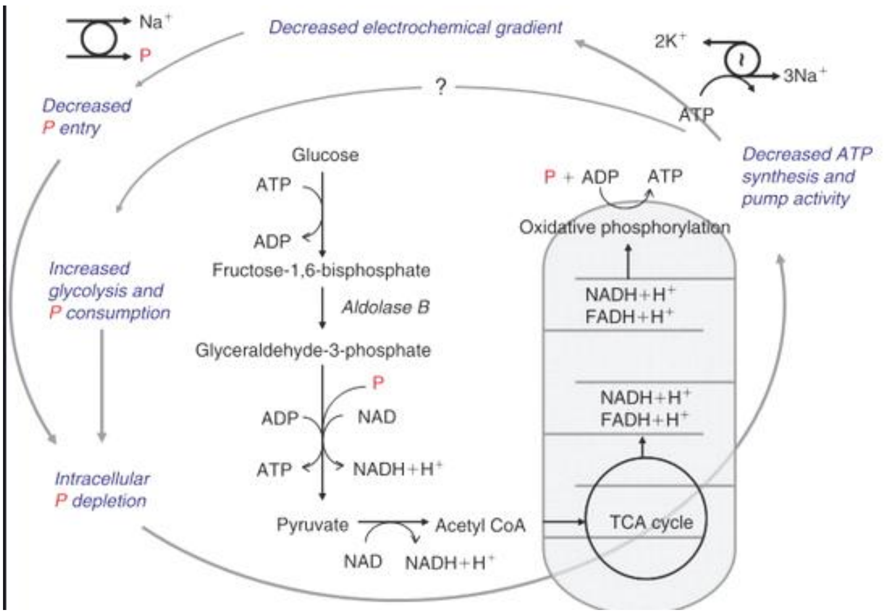


Figure-1 The Crabtree effect.

The **Crabtree effect** describes the phenomenon whereby the yeast, *Saccharomyces cerevisiae*, produces ethanol (alcohol) in aerobic conditions and high external glucose concentrations rather than producing biomass via the tricarboxylic acid (TCA) cycle, the usual process occurring aerobically in most yeasts e.g. *Kluyveromyces* spp.. This phenomenon is observed in most species of the *Saccharomyces*, *Schizosaccharomyces*, *Debaryomyces*, *Brettanomyces*, *Torulopsis*, *Nematospora*, and *Nadsonia* genera. Increasing concentrations of glucose accelerates glycolysis (the breakdown of glucose) which results in the production of appreciable amounts of ATP through substrate-level phosphorylation (Fig. 1). This reduces the need of oxidative phosphorylation done by the TCA cycle via the electron transport chain and therefore decreases oxygen consumption. The crabtree effect works

by repressing respiration by the fermentation pathway, dependent on the substrate. The occurrence of alcoholic fermentation is not primarily due to a limited respiratory capacity, but could be caused by a limit in the cellular Gibbs energy dissipation rate (Fig.2).

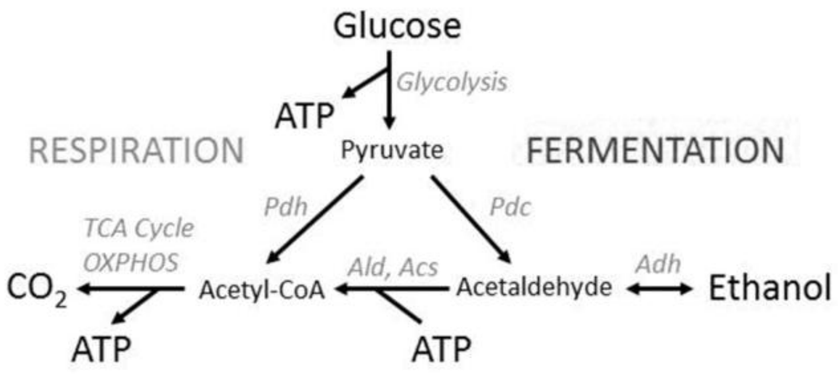


Figure-2: Yeast metabolism.

Brewer’s yeast will undergo fermentation even in the presence of oxygen through a phenomenon we call the Crabtree effect. This effect is a metabolic regulatory mechanism that represses the respiration pathway if fermentable sugars are available for fermentation. Knowing this, we can say for sure that acetic acid flaws in beer are not due to yeast respiration but instead to the metabolic products of Acetobacteria, which are using oxygen to respire – this is a whole different problem (these microbes oxidize ethanol to acetic acid to generate energy). Therefore, not only can oxygen be present, it should be present to ensure that happy, healthy yeast are pitched and sustained in batch fermentation.

Yeast will not die in the absence of oxygen, but in the long run yeast will significantly benefit from the presence of oxygen. Oxygen allows yeast to undergo biosynthesis of unsaturated fatty acids and sterols. These two macromolecules are major components of yeast cell membranes, and their synthesis promotes budding and general well-being of strong, efficient cells. Once cells are depleted of sterols yeast growth will halt. Furthermore, weak cells are prone to get stuck in fermentation, which can lead to more problems that impart a variety of off-flavors in your beer. Viability and vitality of cells are crucial in flawless fermentation.

There are a variety of microorganisms involved in aerobic fermentation. Organism requires oxygen for its energy metabolism and the product will be synthesized. Industrially produced enzyme and antibiotics are the best examples of aerobic fermented products. Those organisms are obligate aerobes, it require high amount of oxygen for their growth and energy production. Hence, the fermenters usually have the aeration provitions such as aerator, impellors and stirrers. The air lift fermenter is the best choice for majority of aerobic fermentations. The bubble column reactor, and the stirrer tank fermenters can be operated either batch wise or continuously to produce large amount of desirable products. Some important products produced industrially b aerobic fermentation is given in table-1.

Table- 1Aerobic fermented products.

Product	Microorganisms
Antibiotics	Streptomyce sp.
Lactic acid	Lactobacillus sp
Citric acid	Aspergillus niger
Alkaloids	Bacillus sp
Bacteriocins	Bacillus sp. Enterococcus and Lactobacillus sp
Enzymes	Bacillus, Pseudomonas, Aspergillus sp. etc

Advantages

1. Slow process, produce low amount of product at the trophophase.
2. Less toxic end products.
3. Low metabolic intermediates.
4. Low pressure to the fermeting organisms.
5. High energy metabolism.
6. Mostly secondary metabolites.
7. Continuous mode of fermentation is possible.

Disadvantages

1. Require costly fermenter with aeration and agitation provisions.
2. Oxygen limitation affects the cell growth and product conversion.
3. High contamination possibilities.
4. Restricted volume of fermenter is used with high power consumption.
5. High labor intensive

8.4 ANAEROBIC FERMENTATION

Fermentation process carried out in the absence of oxygen is called as anaerobic fermentation. There are two types of anaerobic microorganisms viz, obligate anaerobic microorganisms and facultative anaerobic microorganisms. The former like *Clostridium* sp. cannot withstand oxygen or remain active only in the absence of oxygen.

They remain active in the absence of oxygen and produce optimum amount of the desired product. The facultative anaerobes like lactic acid bacteria are able to withstand small amount of oxygen. However, certain organisms like yeast require an initial aeration to build up high cell yield before anaerobic conditions are created. Anaerobic conditions in the fermenter are created either by withdrawing the oxygen present in the head space by an exhaust pump and pumping some inert gases like nitrogen, argon etc. or by flushing it out, by the emergence of certain gases like carbon dioxide or hydrogen

Anaerobic fermentation occurs in the fermentation vessel once the oxygen is discharged and replaced with N_2, CO_2, or another by-product of the fermentation process. Anaerobic fermentation is usually a slower process. In the mid-1850s, the French chemist Louis Pasteur produced anaerobiosis by boiling the medium to drive out oxygen and then introducing inert gas for cultivation. He showed that a microorganism, probably *Clostridium butyricum* was responsible for butyric acid fermentation. In the 1960s and 1970s, anaerobic chambers were invented that allowed the cultivation of numerous anaerobic cultures for certain strictly anaerobic organisms, including *C. botulinum*. During World War I, industrial anaerobic fermentation was further demonstrated by Perkins and Weizmann, who worked on acetonebutanol-ethanol (ABE) fermentation with *C. acetobutylicum*.

Anaerobes may grow under the unfavorable conditions used to minimize contamination during fermentations because they have unusual enzymes and catabolic pathways. Most anaerobic fermentations require little energy to keep cells in suspension. Because less biomass is produced in anaerobic fermentations, more carbon can be converted to the end product, and a higher product yield is attained. Anaerobes can utilize a wide range of substrates, including agricultural waste streams. This reduces the overall cost of the fermentation process. Anaerobic fermentation has been applied to many important

industrial fermentations, such as ethanol production by yeasts, lactic acid preservation of foods, anaerobic digestion of organic matters in ruminant cultivation and waste treatment. The most important industrial fermentation is the anaerobic production of ethanol by *S. cerevisiae* and other yeasts.

However, mixed-culture processes in anaerobic fermentation are also difficult to study and model. The microbial communities are usually unstable, varying with environmental changes and the availability of nutrients. Obligate anaerobes need specialized media and apparatus. They are deactivated by exposure to oxygen. Hence, special skills and meticulous methods are required for the cultivation and manipulation of strictly anaerobic microorganisms.

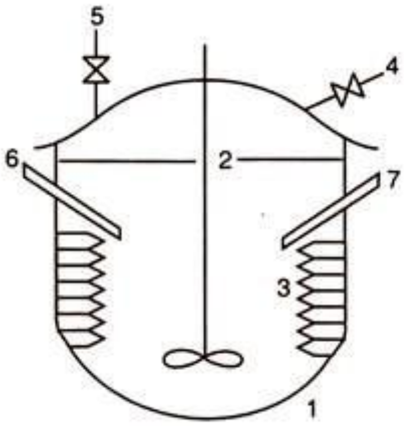


Figure-3: Anaerobic bioreactor.

Stationary medium and viscous medium also creates anaerobic conditions. Sometimes in order to create anaerobic condition, medium is inoculated at the bottom of the fermenter soon after sterilization.

a) Advantages:

1. Production of economically valuables byproducts like carbon dioxide and hydrogen gas during anaerobic fermentation, which may fetch some profits to the manufacturers.
2. Minimum biomass with large quantity of product.
3. Maximum substrate is converted to product.
4. Low contamination possibility.

b) Disadvantages

1. Manufacturers may have to spend more money in providing extra provisions to the fermenter like exhaust pump in order to enforce anaerobic conditions.

2. It requires special media like viscous media whose preparation requires certain costly chemicals.
3. Identification of contaminant is not easy.

8.5 DUEL FERMENTATION

It is a method of continuous product formation using at least two continuous fermentation units and a microorganism capable of being induced, in response to environmental conditions, to undergo a genetic alteration from a state favoring microorganism growth to a state favoring product production by the microorganism. The first continuous fermentation unit is maintained at environmental conditions selected to favor growth of the microorganism and to be nonpermissive for the genetic alteration. The microorganism is grown continuously in the first unit, and a portion of the growing microorganism cell mass is transferred via connecting means to the second continuous fermentation unit. Either the connecting means or the second unit is maintained at second environmental conditions selected to effect the genetic alteration. The altered microorganism is cultured in the second unit. Exudate from this second fermentor (containing microorganism mass and medium) is continually removed and the product which is present, either in the microorganisms themselves or the medium surrounding them, is extracted.

Production of aliphatic acids by fermentation has traditionally been attempted using one of two processes. Propionic and lactic acids have been produced as their calcium salts by fermentation of carbohydrate in the presence of calcium carbonate. After removal of bacterial cells, the undissociated acid was then recovered by acidification of the fermentation broth with sulfuric acid; this acidification resulted in formation of large quantities of gypsum which was separated from the product acid and discarded.

8.5.1 MULTISTAGE CONTINUOUS CULTURE

Multistage continuous fermentation is a continuous fermentation reques several stages of fermentation or multiple organisms require to complete the product formation. In this case several fermenting vessels are inter connected one-by-one and the fermentation process is also carred out in different stages. The primary organism may not utilize the raw material used for the fermentation so, it require pre treatment

process. Microbe mediated pre-treatment is thenewer approach by simplifying the substrate or to make available substrate for the desirable product fermentation. Recently, production of ethanol from cellulosic raw materials require either enzymatic conversion of the cellulose waste in to simple sugars like sugar syrups (glucose, fructose and galactase) or microbial pretreatment for the hydrolysis of compled carbohydrate in to a simple sugars. This process is also carried out in separate fermentation vessel. On the other hand, some metabolite production requires finishing reaction or final polishing step in separate fermentation condition. Hence duel or multistage continuous fermentation process is used.

For example, one technology that is significantly changing industrial ethanol production is very -high - gravity (VHG) fermentation. In VHG fermentation, mashes with greater than 27 g dissolved solids /100 g mash can be batch - fermented with all substrates present at zero time and without the use of conditioned or genetically modified Saccharomyces yeasts. This technology has led to production of 23.8% v/v ethanol in the laboratory from wheat mash containing 38% w/v dissolved solids, and it is gradually being applied to industry where goals of 15–16% or more alcohol in some locations are being set in order to lower costs. In brewing, VHG technology has been successfully tested in pilot plants to produce beers with higher alcohol content which, according to sensory panels, were of remarkably acceptable quality when diluted to the alcohol concentration of commerce.

The figure -4 indicates diagram of a cell recycling multi-stage continuous fermentation system. in which the loss of cell mass is retained by recycling of the biomass in to the reactor. This system improves the rate of fermentation. The first fermenter is inoculated with fresh medium, microbial cell mass is increased in the first vessel and no product or very less quantity of product only fermented in this vessel, following fermenters the end product can be synthesized, and the fresh media replenish the nutrients in the vessels 2 and so on. hence the lass of microbial biomass will be occur, which may be adjusted with concentrated biomass is return to the fermenter.

NOTES

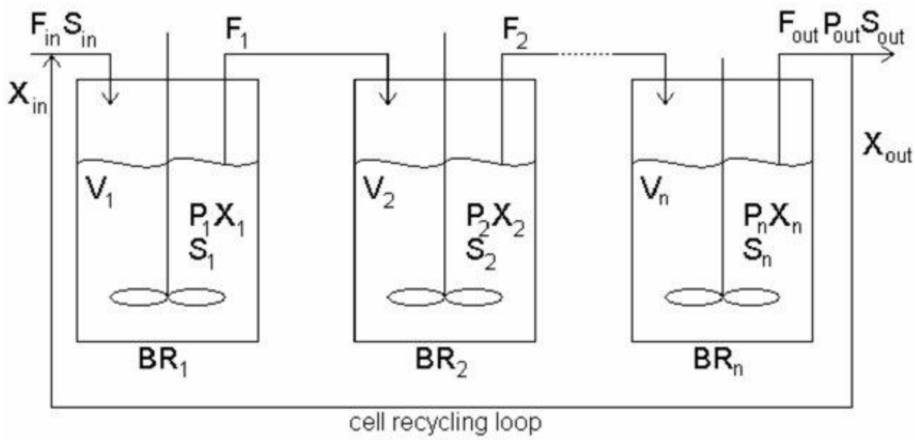


Figure- 4: Diagram of a cell recycling multi-stage continuous fermentation system. (BR n : bioreactor number n; F n : feed flow rate from vessel; P n : product concentration; S n : substrate concentration; V n : volume in vessel; X n : biomass concentration)

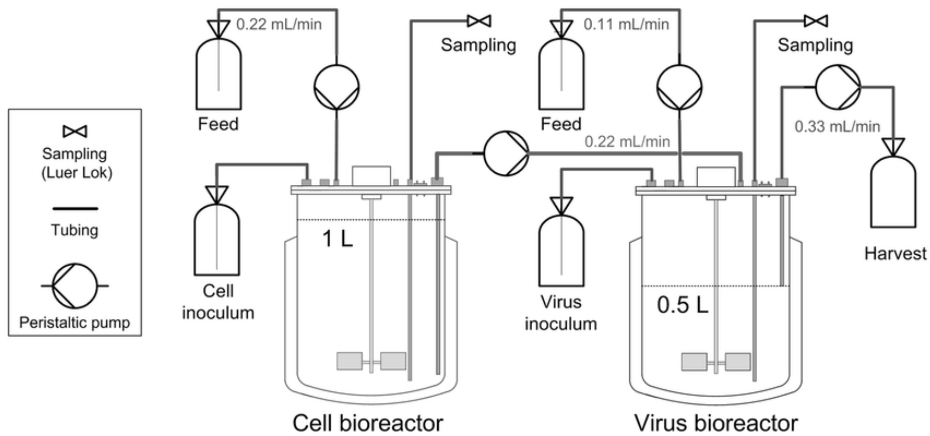


Figure 5: Overview of two-stage bioreactor setup for continuous virus propagation.

In fig.5, AGE1.CR cells are cultivated in two bioreactors. At time of infection, the influenza strain A/Puerto Rico/8/34 was added to the virus bioreactor at a multiplicity of infection of 0.025. Subsequently, the cell concentration in the cell bioreactor was kept at approx. 4–5 x 10^6 cells/mL and cells were constantly fed into the virus bioreactor (feeding rates are depicted). Trypsin was added either to the feed or directly into the virus bioreactor. All green components correspond to the cell bioreactor, all red components to the virus bioreactor. Both reactors are connected via the purple tubing.

Advantages

1. Reduces pretreatment cost
2. Reduces the contamination possibility
3. High yield
4. Waste minimization etc.
5. Less labor.

Disadvantages

High cost investment with high containment facility.

Check your Progress -1

Note: Write your answer in the space given below

a. Give the applications of aerobic fermentation.

b Name any two metabolites produced via anaerobic fermentation.

…………………………………………………………………………

8.6 LET US SUM UP

In this chapter, you have learned and gaining the knowledge of types of microbial fermentation, aerobic and anaerobic process. Duel and multi stage fermentation with their advantages and disadvantages.

8.7 UNIT - END EXERCISES

1. Discuss about aerobic fermentation
2. Discuss about anaerobic fermentation.
3. Explain the importance of multistage fermentation.

8.8 ANSWERS TO CHECK YOUR PROGRESS

a. Slow process, produce low amount of product at the trophophase. Less toxic end products. Low metabolic intermediates. Low pressure to the fermeting organisms. High energy metabolism.

b. Ethanol, butanol acetone etc.

8.9 SUGGESTED READINGS

1. Otero, J. M., & Nielsen, J. (2010). Industrial systems biology. *Biotechnology and Bioengineering*, *105*(3), 439–460.
1. Reader, R. A. (2013). FDA biopharmaceutical product approvals and trends in 2012. *BioProcess International*, *11*(3), 18–27.
2. Van Dien, S. (2013). From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. *Current Opinion in Biotechnology*, *24*, 1061–1068.

3. van Wezel, G. P., & Mcdowall, K. M. (2011). The regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances. *Natural Product Reports*, *28*, 1311–1333.

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NOTES

UNIT -IX: DOWNSTREAM PROCESSING

Structure

9.1 INTRODUCTION

Downstream processing refers to the recovery and the purification of biosynthetic products, particularly pharmaceuticals, from natural sources such as animal or plant tissue or fermentation broth, including the recycling of salvageable components and the proper treatment and disposal of waste. In this chapter, importance of downstream processing in industrial fermentation, processes, problems and requirements of bioproduct recovery and purification are discussed.

1.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the concept of downstream processing
- Know the problems in downstream processing
- Understand the basic requirements of bioproduct recovery and purification.
- Value the role of downstream processing in bioproduct production.

9.3 IMPORTANCE OF DOWNSTREAM PROCESSING

Downstream processing is the recovery and the purification of desired products from the upstream biomass. It refers to the purification of biosynthetic products from their crude preparation like tissue fluids or fermentation broth. In downstream processing, the products which are desired are purified and separated through fermentation or enzyme reactions. The desired products are temperature sensitive, have multiple items to separate, in aqueous medium they are diluted, may have

properties similar to contaminants. Downstream processing is usually considered a specialized field in biochemical engineering, itself a specialization within chemical engineering, though many of the key technologies were developed by chemists and biologists for laboratory scale separation of biological products. Downstream processing and analytical bioseparation both refer to the separation or purification of biological products, but at different scales of operation and for different purposes. Downstream processing implies manufacture of a purified product fit for a specific use, generally in marketable quantities, while analytical bioseparation refers to purification for the sole purpose of measuring a component or components of a mixture, and may deal with sample sizes as small as a single cell.

The extraction and purification of fermentation products may be difficult and costly. Ideally, one is trying to obtain a high-quality product as quickly as possible at an efficient recovery rate using minimum plant investment operated at minimal costs. Unfortunately, recovery costs of microbial products may vary from as low as 15% to as high as 70% of the total manufacturing costs. The chosen process, and therefore its relative cost, will depend on the specific product. The indicate percentage of total costs being 15% for industrial ethanol, 20–30% for bulk penicillin G and up to 70% for enzymes.

The extraction and purification of products such as recombinant proteins and monoclonal antibodies can account for 80–90% of the total processing costs. The high (and sometimes dominant) cost of downstream processing will affect the overall objective in some fermentation. If a fermentation broth is analyzed at the time of harvesting, it will be discovered that the specific product may be present at a low concentration (typically 0.1–5 g dm^{-3}) in an aqueous solution that contains intact microorganisms, cell fragments, soluble and insoluble medium components, and other metabolic products. The product may also be intracellular, heat labile, and easily broken down by contaminating microorganisms. All these factors tend to increase the difficulties of product recovery.

To ensure good recovery or purifiation, speed of operation may be the overriding factor because of the labile nature of a product. The processing equipment therefore must be of the correct type and also the correct size to ensure that the harvested broth can be processed within a satisfactory time limit. It should also be noted that each step or unit

operation in downstream processing will involve the loss of some product as each operation will not be 100% efficient and product degradation may have occurred. Even if the percentage recovery for each step is very high, say for example, 90%, after five steps only around 60% of the initial product will be obtained. Hence, it is also important that the minimum number of unit operations possible is used to maximize product recovery.

Numerous numbers of important products have been isolated and purified through downstream processing. These have helped make significant steps especially in the field of pharmaceutics.

Purification and recovery of biosynthetic products, mostly pharmaceutics from sources hat are natural. For example, plant and animal tissues or fermentation froth.

1. Specific roles of Recycling of components that can be salvaged from waste.
2. Proper waste disposal and treatment
3. Good downstream processing

Downstream processing is very important in the manufacture of antibiotics such as penicillin. The process is applied in the purification and separation of antibiotics from a number of mediums. This is not an easy task bearing in mind that antibiotics' concentration may be very low in the solutions in question. Again, they can be applied in large scale manufacture of monoclonal antibodies (mAbs). Since antibody therapies are characterized by long-term administration of large antibody does, biopharmaceutical companies greatly appreciate down processing for facilitating industrial manufacture of antibodies.

A number of hormones are also resultant products of downstream processing. Some of the most commonly produced hormones include growth hormone and insulin. Follicle Stimulating Hormone is also another example.

Various research projects have also seen to the application of downstream processing in the manufacture of important vaccines. Common examples include vaccines for influenza and small pox. Moreover, downstream process is also applied in industrial manufacture of enzymes. Enzymes are normally synthesized by living cells and are responsible for triggering chemical reactions. Industrial production of enzymes is therefore very important since they are

appliedin processes such as food preservation and processing, manufacture of textiles, paper industry, scientific research, etc. A good example is a fungal amylase enzyme known as taka-diastate. This was the first enzyme to be produced in industry (U.S.A 1896) and was used pharmaceutically as an agent for treating digestive disorders.

9.4 PROBLEM AND REQUIREMENT OF BIOPRODUCT RECOVERY AND PURIFICATION

The recovery of enzymes from natural extracts on a large scale through bioseparation processes, for various applications, is still a challenging task in the current trend. The problems associated with the recovery of enzymes are the process steps and production costs that form a barrier for large scale production industries. About 80% of the production cost dwells in the downstream process recovery. Several researchers have developed methodologies for the protein and enzyme extraction using various extraction techniques. New process designs are needed to improve the existing process techniques associated with enzyme extraction.

9.4.1 BIOSEPARATION AND ITS SELECTIVITY

The incredible developments in Biotechnology have extended its hands in several fields like industrial usage, research, clinical, etc. Heavy demand for highly valued biomolecules have led to an evolution in the field of Biotechnology through the application of microbial fermentation techniques, genetically modified organisms and natural sources. Therapeutic proteins, recombinant products and high complicated products like naturally derivatized enzymes prompt for sensitive and highly specific separation protocols which demands high cost and process time.

Downstream processing plays an inevitable role in any product advancement because the overall recovery of proteins prominently decides the production cost. The downstream processing techniques are an unavoidable step in the protein purification industry as the high value biomolecules focus on accurate and appropriate extraction protocol.

Generally, four stages were followed in protein recovery which incorporates a sequence of unit operations. 1) Harvesting the cell

biomass; 2) Separation/ extraction of the product; 3) Purification of the product 4) Concentration or drying, and 5) Marketing.

Design standards and kinetic assessments were the major factors in unit operations for both laboratory and large scale process. The target was achieved by carefully customizing the set of unit operations and providing it as a successful protocol. The client and investor focuses on yield and purity which are significant terms in downstream processing as the 80% of the production cost depends on the extraction and purification. In protein recovery, the conventional filtration techniques restrict the yield whereas column chromatography and precipitation process are expensive and faces scale up problems. An eco-friendly, efficient, economical and effective process technique is much desired. The predominant role of an engineer is to propose a protocol with limited process steps that satisfies yield and purity and also be in economical.

Bioseparation is mainly applied for the isolation and purification of high value biomolecules from its crude sources. The bioproducts can be derived from various categories of raw sources viz. Fermentation broth, metabolite intermediates from natural extract, recycle stream, etc.

The complex and sensitive nature of these sources does not facilitate the separation through single, simple extraction process. So a step by step protocol for product separation is introduced through RIPP concept, where R denote Removal of soluble, I indicates isolation of product, P refers to purification of product and P represent polishing of product. Each process step has its own set of operations and their selective criteria.

1. The goal of a Biotechnology engineer is to select the minimum process steps to attain the target.
2. Product characterization helps to reduce these trails and also for successful protocol development.
3. The ultimate expectation is the exceptionally pure products with appreciable yield which compete the market demand to satisfy the investors.
4. Ultimately the engineer has to choose the right process, parameter, equipment for the product development within the budget.
5. Complexity of Bioseparation

6. Identifying the most appropriate process parameters is the challenging task in Bioseparation processes.
7. Need for more number of unit operations not only increases the equipment cost but also its ancillaries, maintenance and manpower.
8. Large quantities of effluents produced from the process steps and its disposal/treatments also indirectly influence the process cost.

Since it deals with complex sources, the separation of target protein without losing its bioactivity is very much a tedious process.

9.4.2 NEED OF PROCESS STEP REDUCTION

Affinity separation has emerged as a separate era in downstream processing. Process integration is another concept in bioseparation which finds many applications for protein extraction from its natural sources. Application of chromatographic techniques at the purification step raises the purity fold which in turn increases the operation cost due to its high specific condition monitoring and other maintenance (installation cost, matrix cost). In order to reduce the process cost to at least about 30-40%, a chromatograph free operation that generates result equivalent to chromatographic technique is needed.

9.4.3 SINGLE STEP PURIFICATION

Bioseparation involves a sequence of unit operation which helps to resolve the actual objective of downstream processing (recovery, Isolation, Purification and Polishing). A minimum of two to three steps were required to resolve these objective at each stage.

For example, in the recovery operation excluding pretreatment the steps involved are centrifugation and filtration.

1. In isolation operation, membrane filtration and extraction steps were carried out.
2. Each unit operation requires one or two ancillary operations like buffer treatment, cell wash, backward extraction, retentive recycling, solvent recovery and waste effluent disposal.
3. This ancillary operation not only affects the process cost but also consumes process time and labor.

In order to overcome the above complications, a cutting edge technology in high demand is expected to resolve all the problems. A Single Step Separation (SSS) is proposed to solve minimum two

operations in single step (separation/ Purification or Isolation/ Purification) at minimal process cost and maintenance.

9.4.4 AQUEOUS TWO PHASE EXTRACTION

The use of organic solvents in the extraction process failed to give non denatured outcomes, high pure outputs and product recovery. The mode of action in organic extraction has inherently tough operating conditions and is highly reactive with the biological products. A biocompatible, mild, protein friendly, maintenance free, easily scalable system with low solvent cost is required for separating biomolecules. In bioseparation, an aqueous two phase (ATP) system is a proven technique that satisfies all the above requirements and extracts all types of biomolecules such as proteins, enzymes, antibodies, antibiotics, DNA, RNA and amino acids, etc.

Addition of two-water soluble polymers or a polymer and salt to aqueous medium above their critical concentration leads to the formation of ATP by which two phases are formed without the need for an organic solvent. The two immiscible phases are highly biocompatible and are clearly separated/ defined with good interfacial tension, electrostatically interactive and hydrophobic in nature. The widely used polymer/salt system is polyethylene glycol/potassium phosphate, and polyethylene glycol/dextran is an extensively used polymer/polymer system. The polymer/salt systems are highly preferable in industrial applications because of the easy preparation, less time requirement, high selectivity of proteins. Moreover, proteins can be extracted from the salt phase using simple dialysis technique. A high selective method to improve the product quality is put forth. An affinity partitioning in aqueous two phase system with the assistance of ligands (protein affinity groups) is proposed. The steps involved in the affinity partitioning comprise the following points for protein extraction.

1. Formation of an affinity macro-ligand by coupling the affinity ligand to a polymeric material and holding of the target protein by the affinity method through affinity macro-ligand.
2. Separation of the covalent linkage from other components.
3. Extraction of the target protein from this complex affinity macro-ligand bonding.

Many protocols based on polymer functionalized, polymer modified, chemically modified affinity partitioning have been reported so far. The process cost and maintenance cost in affinity partitioning are not reduced to an appreciable extent. So a free liganding approach in aqueous two phase system using various ligands was experimented. Very few protocols have been reported so far for the dye ligands, free polymer ligands and for IL adjuvants in the extraction of standard protein. The present study investigates the use of free ligands in aqueous two phase extraction system for natural enzyme extracts and its selection, optimization, thermodynamic perspectives and purity studies.

Check your Progress -1

Note: Write your answer in the space given below

a. What are the basic problems arrived during down stream processing.

b. Give the basic steps of product recovery?

…………………………………………………………………………

9.5 LET US SUM UP

In this unit, you have learned about the downstream processing, the recovery and the purification of biosynthetic products, from natural sources such as animal or plant tissue or fermentation broth, including the recycling of salvageable components and the proper treatment and disposal of waste. In this chapter, importance of downstream processing in industrial fermentation, processes, problems and requirements of bioproduct recovery and purification are discussed. This knowledge help you to understand the basic problems during the recovery of products.

9.6 UNIT - END EXERCISES

1. List the basic problems of downstream processing.
2. List the importance of downstream processing.

9.7 ANSWERS TO CHECK YOUR PROGRESS

1. Biomass of the fermented media, multiple products, cell lysate , toxic waste etc are raising the problem. Solubility of the product is also important for product recovery.
2. Harvesting the biomass, separation / extraction of the product, Purification and concentration of the product, finally drying.

NOTES

9.8 SUGGESTED READINGS

1. Otero, J. M., & Nielsen, J. (2010). Industrial systems biology. *Biotechnology and Bioengineering*, *105*(3), 439–460.
1. Reader, R. A. (2013). FDA biopharmaceutical product approvals and trends in 2012. *BioProcess International*, *11*(3), 18–27.
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UNIT -X: DOWNSTREAM PROCESSING-II

Structure

10.1 INTRODUCTION

In this chapter, the fermented product recovery methods such as removal solids including cell biomass and other suspended solids by various physical and chemical methods have been discussed. The principle of filteration, precipitation and centrifugations also discussed in detailed.

10.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the concept product recovery and purification
- To know the physical methods of solids removal.
- Understand the foam separation methods.
- Know the techniques such as precipitation, filtration and centrifugation and its use in product recovery.

10. 3 BIO PRODUCT RECOVERY AND PURIFICATION

If a fermentation broth is analyzed at the time of harvesting, it will be discovered that the specific product may be present at a low concentration (typically 0.1–5 g dm^{-3}) in an aqueous solution that contains intact microorganisms, cell fragments, soluble and insoluble medium components, and other metabolic products. The product may

also be intracellular, heat labile, and easily broken down by contaminating microorganisms. All these factors tend to increase the difficulties of product recovery. To ensure good recovery or purification, speed of operation may be the overriding factor because of the labile nature of a product.

The processing equipment therefore must be of the correct type and also the correct size to ensure that the harvested broth can be processed within a satisfactory time limit. It should also be noted that each step or unit operation in downstream processing will involve the loss of some product as each operation will not be 100% efficient and product degradation may have occurred. Even if the percentage recovery for each step is very high, say for example, 90%, after five steps only around 60% of the initial product will be obtained.

Hence, it is also important that the minimum number of unit operations possible is used to maximize product recovery (Fig.1).
The choice of recovery process is based on the following criteria:

1. The intracellular or extracellular location of the product.
2. The concentration of the product in the fermentation broth.
3. The physical and chemical properties of the desired product (as an aid to select separation procedures).

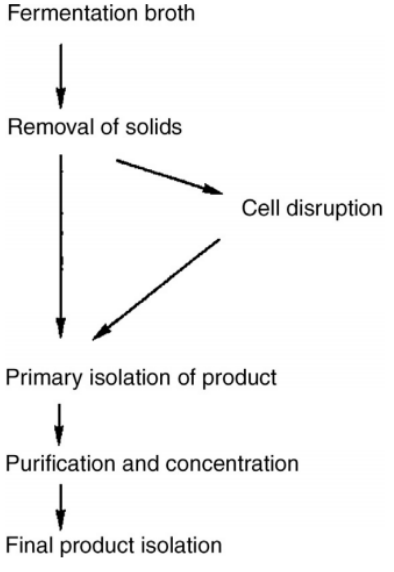


Fig.1 Stages in the Recovery of Product from a Harvested Fermentation Broth.

4. The intended use of the product.
5. The minimal acceptable standard of purity.
6. The magnitude of biohazard of the product or broth.

7. The impurities in the fermenter broth.
8. The marketable price for the product.

It may be possible to modify the handling characteristics of the broth so that it can be handled faster with simpler equipment making use of a number of techniques:

1. Selection of a microorganism which does not produce pigments or undesirable metabolites.
2. Modifiation of the fermentation conditions to reduce the production of undesirable metabolites.
3. Precise timing of harvesting.
4. pH control after harvesting.
5. Temperature treatment after harvesting.
6. Addition of flcculating agents.
7. Use of enzymes to attack cell walls.

Certain antifoams remain in the supernatant and may affect centrifugation, ultra filtration or ion-exchange resins used in recovery stages. Trials may be needed to find the most suitable antifoam. The ionic strength of the production medium may be too high, resulting in the harvested supernatant needing dilution with demineralized water before it can be processed. Such a negative procedure should be avoided if possible by unified research and development programs. Media formulation is dominated by production requirements, but the protein content of complex media should be critically examined in view of subsequent enzyme recovery. When considering water recycle in biomass production. They stated that the interaction between the different unit operations in a recycle process made it imperative that commercial plant design and operation should be viewed in an integrated fashion. Flow sheets for recovery of penicillin, cephamycin C, citric acid, and micrococcal nuclease are given in Figs 2 to illustrate the range of techniques used in microbiological recovery processes. A series of comprehensive flw sheets for alcohols, organic acids, antibiotics, carotenoids, polysaccharides, intra- and extracellular enzymes, single-cell proteins, and vitamins have been produced.

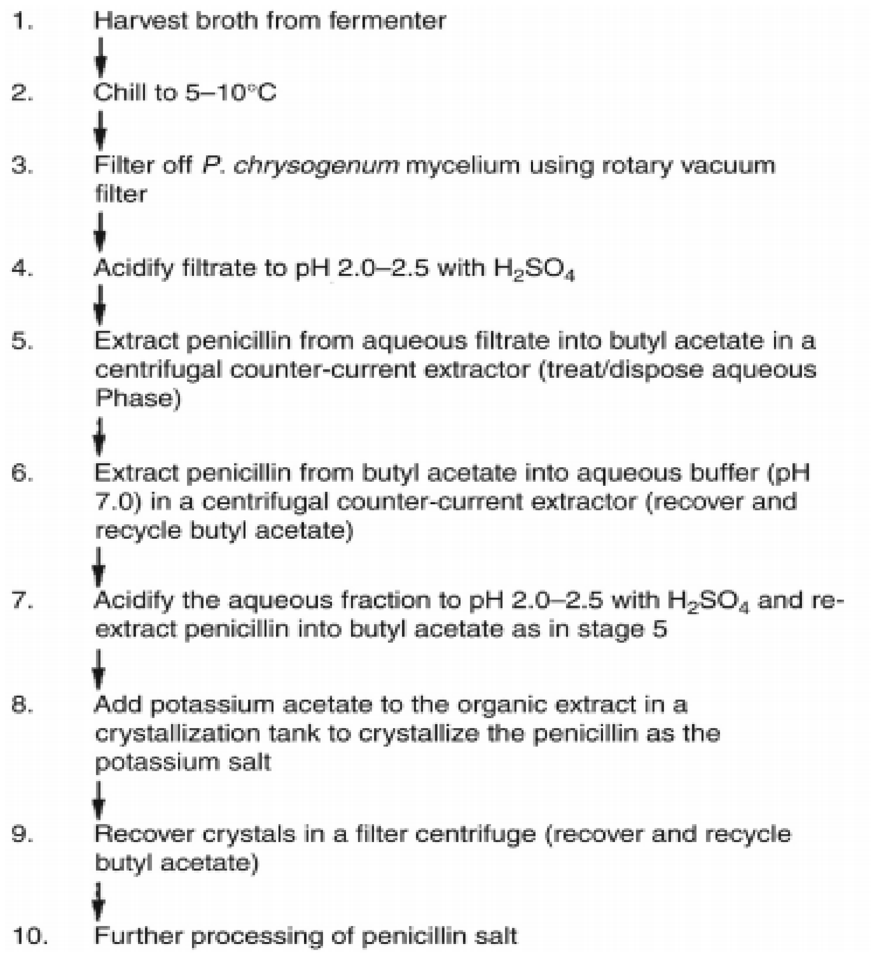


Figure. 2 Recovery and Partial Purification of Penicillin G

In the selection of processes for the recovery of biological products, it should always be understood that recovery and production are interlinked, and that good recovery starts in the fermentation by the selection of, among other factors, the correct media and time of harvesting. The recovery and purifiation of many compounds may be achieved by a number of alternative routes. The decision to follow a particular route involves comparing the following factors to determine the most appropriate under a given set of circumstances:

- Capital costs
- Processing costs
- Throughput requirements
- Yield potential
- Product quality
- Technical expertise available
- Conformance to regulatory requirements

- Waste treatment needs
- Continuous or batch processing
- Automation
- Personnel health and safety

The major problem currently faced in product recovery is the large-scale purification of biologically active molecules. For a process to be economically viable, large-scale production is required, and therefore large-scale separation, recovery, and purification. This then requires the transfer of small-scale preparative/analytical technologies (eg, chromatographic techniques) to the production scale while maintaining efficiency of the process, bioactivity of the product and purity of the product so that it conforms to the safety legislation and regulatory requirements. Developments in this field and remaining areas for development are documented.

10. 4 REMOVAL OF MICROBIAL CELL AND OTHER SOLID MATERIAL

Microbial cells and other insoluble materials are normally separated from the harvested broth by filtration or centrifugation. Because of the small size of many microbial cells, it will be necessary to consider the use of fitter aids to improve filtration rates, while heat and flocculation treatments are employed as techniques for increasing the sedimentation rates in centrifugation. Flocculation can also be utilized in other downstream processing operations to aid product recovery. The use of the flocculants chitosan and polyacrylamide on cell debris and soluble protein in the fermentation broth, to enhance the recovery of 1,3-propanediol by reactive extraction and distillation. The methods of cell and cell debris separation described in the following sections have been practiced for many years. These include the use of electrophoresis and dialect rophoresis to exploit the charged properties of microbial cells, ultrasonic treatment to improve flocculation characteristics and magnetic separations. Although not necessarily for the removal of cells, other downstream operations, which involve the application of an electrical field, are also showing potential. One such process is electro dialysis that involves the transfer of ions from a dilute solution to a concentrated one through a semi permeable membrane by applying an electrical field. The use of electro dialysis in the separation of organic acids in aqueous solution where the product, sodium butyrate, was successfully transferred from the aqueous phase into an

ionic liquid phase through electrodialysis. A recovery rate of 99% was obtained with reduced energy input compared to traditional processing.

10. 5 FOAM SEPARATION

Foam separation depends on using methods, which exploit differences in surface activity of materials. The material may be whole cells or molecular such as a protein or colloid, which is selectively adsorbed or attached to the surface of gas bubbles rising through a liquid, to be concentrated or separated and fially removed. It may be possible to make some materials surface active by the application of surfactants such as long-chain fatty acids, amines, and quaternary ammonium compounds. Materials made surface active and collected are termed colligends whereas the surfactants are termed collectors. When developing this method of separation, the important variables, which may need experimental investigation are pH, air flaw rates, surfactants, and colligend-collector ratios. The recovery of surface active products is clearly an important potential application of this technique. The use of foam separation in the recovery of the lipopeptide biosurfactant surfactin from *B. subtilis* cultures. They report that improved surfactin recovery can be achieved when foaming was simultaneous with the fermentation stage rather than as a nonintegrated semibatch process. The investigated foam separation of *E. coli* starting with an initial cell concentration of 7.2×10^8 cells cm–^{3}. Using lauric acid, stearyl amine, or t-octyl amine as surfactants, it was shown that up to 90% of the cells were removed in 1 min and 99% in 10 min. The technique also proved successful with Chlorella sp. and *Chlamydomonas* sp. In other work with *E. coli*, were able to achieve cell enrichment ratios of between 10 and 1×10^6 using ethyl-hexadecyl-dimethyl ammonium bromide. The effects of a range of both organic and inorganic additives on flatation recovery of *Saccharomyces* cerevisiae. They report that compounds associated with cellular metabolism such as acetate and ethanol can improve flatation recovery of yeast cells.

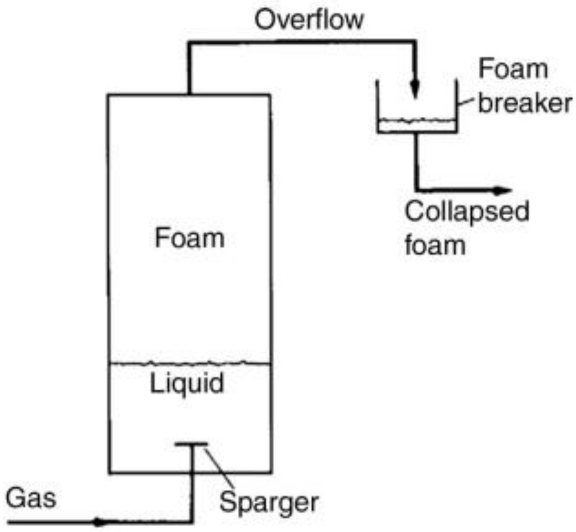


Fig. 3 Schematic Flow Diagram for Foam Fractionation (Wang & Sinskey, 1970)

10. 6 PRECIPTATION

Precipitation may be conducted at various stages of the product recovery process. It is a particularly useful process as it allows enrichment and concentration in one step, thereby reducing the volume of material for further processing. It is possible to obtain some products (or to remove certain impurities) directly from the broth by precipitation, or to use the technique after a crude cell lysate has been obtained. Typical agents used in precipitation render the compound of interest insoluble, and these include:

1. Acids and bases to change the pH of a solution until the isoelectric point of the compound is reached and pH equals pI, when there is then no overall charge on the molecule and its solubility is decreased.
2. Salts such as ammonium and sodium sulfate are used for the recovery and fractionation of proteins. The salt removes water from the surface of the protein revealing hydrophobic patches, which come together causing the protein to precipitate. The most hydrophobic proteins will precipitate fist, thus allowing fractionation to take place. This technique is also termed "salting out."
3. Organic solvents. Dextrans can be precipitated out of a broth by the addition of methanol. Chilled ethanol and acetone can be used in the precipitation of proteins mainly due to changes in the dielectric properties of the solution.

4. Nonionic polymers such as polyethylene glycol (PEG) can be used in the precipitation of proteins and are similar in behavior to organic solvents.
5. Polyelectrolytes can be used in the precipitation of a range of compounds, in addition to their use in cell aggregation.
6. Protein binding dyes (triazine dyes) bind to and precipitate certain classes of protein.
7. Affinity precipitants are an area of much current interest in that they are able to bind to, and precipitate, compounds selectively.
8. Heat treatment as a selective precipitation and purification step for various thermostable products and in the deactivation of cell proteases.

10. 7 FILTRATION

Filtration is one of the most common processes used at all scales of operation to separate suspended particles from a liquid or gas, using a porous medium which retains the particles but allows the liquid or gas to pass through. Gas filtration has been discussed in detail elsewhere. It is possible to carry out filtration under a variety of conditions, but a number of factors will obviously influence the choice of the most suitable type of equipment to meet the specified requirements at minimum overall cost, including:

1. The properties of the filtrate, particularly its viscosity and density.
2. The nature of the solid particles, particularly their size and shape, the size distribution and packing characteristics.
3. The solids: liquid ratio.
4. The need for recovery of the solid or liquid fraction or both.
5. The scale of operation.
6. The need for batch or continuous operation.
7. The need for aseptic conditions.
8. The need for pressure or vacuum suction to ensure an adequate flow rate of the liquid.

10.7.1USE OF FILTER AIDS

It is common practice to use fitter aids when filtering bacteria or other fie or gelatinous suspensions which prove slow to fitter or partially block a fitter. Kieselguhr (diatomaceous earth) is the most widely used material. It has a voidage of approximately 0.85, and, when it is mixed with the initial cell suspension, improves the porosity

of a resulting fiter cake leading to a faster flw rate. Alternatively, it may be used as an initial bridging agent in the wider pores of a fiter to prevent or reduce blinding. The term “blinding” means the wedging of particles which are not quite large enough to pass through the pores, so that an appreciable fraction of the filter surface becomes inactive. The minimum quantity of fiter aid to be used in fitration of a broth should be established experimentally. Kieselguhr is not cheap, and it will also absorb some of the fitrate, which will be lost when the filter cake is disposed. The main methods of using the filter aid are:

1. A thin layer of Kieselguhr is applied to the fitter to form a precoat prior to broth filtration.
2. The appropriate quantity of fiter aid is mixed with the harvested broth. Filtration is started, to build up a satisfactory fitter bed. The initial raffiate is returned to the remaining broth prior to starting the true filtration.
3. When vacuum drum filters are to be used which are filtered with advancing knife blades, a thick precoat fitter is initially built up on the drum.

In some processes such as microbial biomass production, fitter aids cannot be used and cell pretreatment by flocculation or heating must be considered. In addition it is not normally practical to use fitter aids when the product is intracellular and its removal would present a further stage of purification.

10.7.2 PLATE AND FRAME FILTERS

A plate and frame filter is a pressure filter in which the simplest form consists of plates and frames arranged alternately. The plates are covered with filter cloths or filter pads. The plates and frames are assembled on a horizontal framework and held together by means of a hand screw or hydraulic ram so that there is no leakage between the plates and frames, which form a series of liquid-tight compartments. The slurry is fed to the filter frame through the continuous channel formed by the holes in the corners of the plates and frames. The filtrate passes through the filter cloth or pad, runs down grooves in the filter plates and is then discharged through outlet taps to a channel. Sometimes, if aseptic conditions are required, the outlets may lead directly into a pipe. The solids are retained within the frame and filtration is stopped when the frames are completely filled or when the flow of filtrate becomes uneconomically low. On an industrial scale, the plate and frame filter is one of the cheapest filters per unit of

filtering space and requires the least floor space, but it is intermittent in opera- tion (a batch process) and there may be considerable wear of filter cloths as a result of frequent dismantling. This type of filter is most suitable for fermentation broths with a low solids content and low resistance to filtration. It is widely used as a "polishing" device in breweries to filter out residual yeast cells following initial clarification by centrifugation or rotary vacuum filtration. It may also be used for collecting high value solids that would not justify the use of a continuous filter. Because of high la- bor costs and the time involved in dismantling, cleaning, and reassembly, these filters should not be used when removing large quantities of worthless solids from a broth.

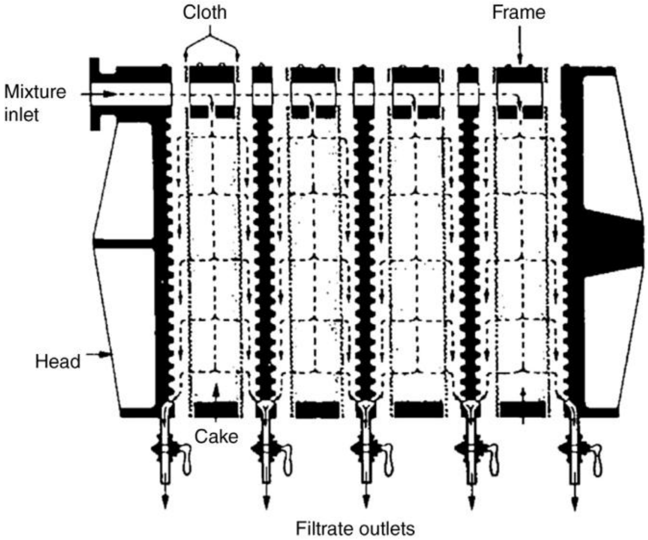
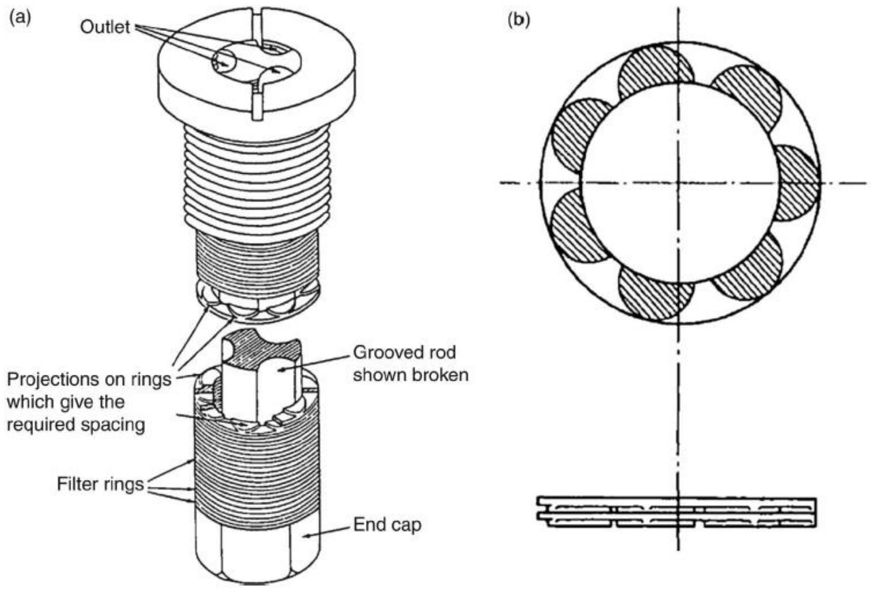


Figure-4: Plate and frame filter assembly

10.7.3 PRESSURE LEAF FILTERS

There are a number of intermittent batch filters usually called by their trade names. These filters incorporate a number of leaves, each consisting of a metal framework ofgrooved plates, which is covered with a fine wire mesh, or occasionally a filter cloth and often precoated with a layer of cellulose fibers. The process slurry is fed into the filter, which is operated under pressure or by suction with a vacuum pump. Because the filters are totally enclosed it is possible to sterilize them with steam. This type of filter is particularly suitable for "polishing" large volumes of liquids with low solids content or small batch filtrations of valuable solids.



(a) Metafilter pack (Coulson & Richardson, 1991). (b) Rings for metafilter (Coulson & Richardson, 1991)

Figure- 5: Metafilters.

10.7.4 VERTICAL METAL-LEAF FILTER

This filter consists of a number of vertical porous metal leaves mounted on a hollow shaft in a cylindrical pressure vessel. The solids from the slurry gradually build up on the surface of the leaves and the filtrate is removed from the plates via the horizontal hollow shaft. In some designs the hollow shaft can be slowly rotated during filtration. Solids are normally removed at the end of a cycle by blowing air through the shaft and into the filter leaves.

10.7.5 HORIZONTAL METAL-LEAF FILTER

In this filter, the metal leaves are mounted on a vertical hollow shaft within a pressure vessel. Often, only the upper surfaces of the leaves are porous. Filtration is continued until the cake fills the space between the disc-shaped leaves or when the operational pressure has become excessive. At the end of a process cycle, the solid cake can be discharged by releasing the pressure and spinning the shaft with a drive motor.

10.7.6 STACKED-DISC FILTER

One kind of filter of this type is the Metafilter. This is a very robust device and because there is no filter cloth and the bed is easily replaced, labor costs are low. It consists of a number of precision-made

rings, which are stacked on a fluted rod. The rings (22 mm external diameter, 16 mm internal diameter, and 0.8 mm-thick) are normally made from stainless steel and precision stamped so that there are a number of shoulders on one side. This ensures that there will be clearances of 0.025–0.25 mm when the rings are assembled on the rods. The assembled stacks are placed in a pressure vessel, which can be sterilized if necessary. The packs are normally coated with a thin layer of Kieselguhr, which is used as a filter aid. During use, the filtrate passes between the discs and is removed through the grooves of the fluted rods, while solids are deposited on the filter coating. Operation is continued until the resistance becomes too high and the solids are removed from the rings by applying back pressure via the fluted rods. Metafilters are primarily used for "polishing" liquids such as beer.

10.7.7 CROSS-FLOW FILTRATION (TANGENTIAL FILTRATION)

In the filtration processes previously described, the flow of broth was perpendicular to the filtration membrane. Consequently, blockage of the membrane led to the lower rates of productivity and/or the need for filter aids to be added, and these were serious disadvantages. In contrast, an alternative which is rapidly gaining prominence both in the processing of whole fermentation broths is cross-flow filtration. Here, the flow of medium to be filtered is tangential to the membrane, and no filter cake builds up on the membrane. The benefits of cross-flow filtration are:

1. Efficient separation, >99.9% cell retention.
2. Closed system; for the containment of organisms with no aerosol formation
3. Separation is independent of cell and media densities, in contrast to centrifugation.
4. No addition of filter aid.

The major components of a cross-flow filtration system are a media storage tank (or the fermenter), a pump, and a membrane pack. The membrane is usually in a cassette pack of hollow fibers or flat sheets in a plate and frame type stack or a spiral cartridge. In this way, and by the introduction of a much convoluted surface, large filtration areas can be attained in compact devices. Two types of membrane may be used; microporous membranes (microfiltration) with a specific pore size (0.45, 0.22 µm etc.) or an ultrafiltration membrane with a specified molecular weight cut-off (MWCO). The type of membrane chosen is

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carefully matched to the product being harvested, with microporous and 100,000 MWCO membranes being used in cell separations.

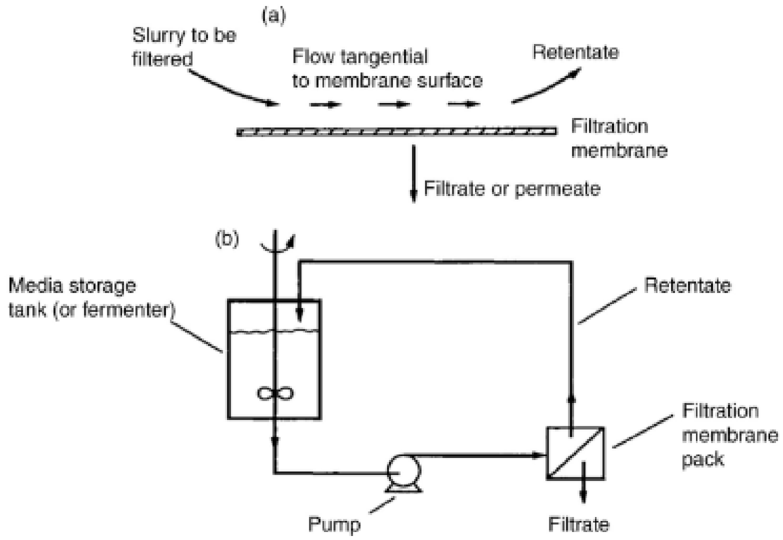


Fig.7 Schematic diagram of cross-flow filtration. (b) Major components of a cross-flow filtration system

10/7.7 ROTARY VACUUM FILTERS

Large rotary vacuum filters are commonly used by industries, which produce large volumes of liquid which need continuous processing. The filter consists of a rotating, hollow, segmented drum covered with a fabric or metal filter, which is partially immersed in a trough containing the broth to be filtered (Fig. 7). The slurry is fed on to the outside of the revolving drum and vacuum pressure is applied internally so that the filtrate is drawn through the filter, into the drum and finally to a collecting vessel. The interior of the drum is divided into a series of compartments, to which the vacuum pressure is normally applied for most of each revolution as the drum slowly revolves (~1 rpm). However, just before discharge of the filter cake, air pressure may be applied internally to help ease the filter cake off the drum. A number of spray jets may be carefully positioned so that water can be applied to rinse the cake. This washing is carefully controlled so that dilution of the filtrate is minimal.

It should be noted that the driving force for filtration (pressure differential across the filter) is limited to 1 atmosphere (100 kN m–2) and in practice it is significantly less than this. In contrast, pressure filters can be operated at many atmospheres pressure. A number of

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rotary vacuum drum filters are manufactured, which differ in the mechanism of cake discharge from the drum:

1. String discharge.
2. Scraper discharge.
3. Scraper discharge with precoating of the drum.

String discharge

Fungal mycelia produce a fibrous filter cake, which can easily be separated from the drum by string discharge (Fig. 7). Long lengths of string 1.5 cm apart are threaded over the drum and round two rollers. The cake is lifted free from the upperpart of the drum when the vacuum pressure is released and carried to the small rollers where it falls free.

Scraper discharge

Yeast cells can be collected on a filter drum with a knife blade for scraper discharge (Fig. 7). The filter cake which builds up on the drum is removed by an accurately positioned knife blade. Because the knife is close to the drum, there may be gradual wearing of the filter cloth on the drum.

Scraper discharge with precoating of the drum

The filter cloth on the drum can be blocked by bacterial cells or mycelia of actinomycetes. This problem is overcome by precoating the drum with a layer of filter-aid 2–10 cm thick. The cake which builds up on the drum during operation is cut away by the knife blade (Fig. 7), which mechanically advances toward the drum at a controlled slow rate. Alternatively, the blade may be operated manually when there is an indication of "blinding" which may be apparent from a reduction in the filtration rate. In either case the cake is removed together with a very thin layer of precoat. The operating variables studied include drum speed, extent of drum submergence, knife advance speed, and applied vacuum. The work indicated that optimization for a new process might require prolonged trials. Although primarily used for the separation of microorganisms from broth, studies have indicated that rotary vacuum filters can be effective in the processing of disrupted cells.

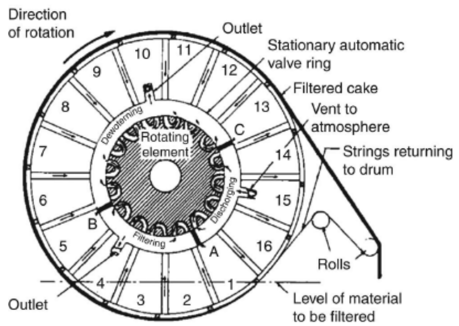
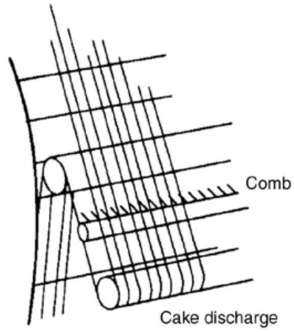
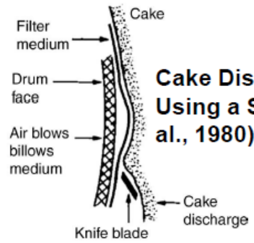


Diagram of String-Discharge Filter Operation
Sections 1 to 4 are filtering; sections 5 to 12 are dewatering; and section 13 is discharging the cake with the string discharge. Sections 14, 15 and 16 are ready to start a new cycle. A, B and C represent dividing members in the annular ring (Miller et al., 1973).



Cake Discharge on a Drum Filter Using Strings



Cake Discharge on a Drum Using a Scraper (Talcott et al., 1980)

Figure-7: Rotary vacuum filter.

10. 8 CENTRIFUGATION

Microorganisms and other similar sized particles can be removed from a broth by using a centrifuge when fitration is not a satisfactory separation method. Although a centrifuge may be expensive when compared with a fiter it may be essential when:

1. Filtration is slow and difficult.
2. The cells or other suspended matter must be obtained free of filter aids.
3. Continuous separation to a high standard of hygiene is required.

Noncontinuous centrifuges are of extremely limited capacity and therefore not suitable for large-scale separation. The centrifuges used in harvesting fermentation broths are all operated on a continuous or semicontinuous basis. Some centrifuges can be used for separating two immiscible liquids yielding a heavy phase and light phase liquid, as well as a solids fraction. They may also be used for the breaking of emulsions.

10.8.1 RANGE OF CENTRIFUGES

A number of centrifuges will be described which vary in their manner of liquid and solid discharge, their unloading speed and their relative maximum capacities. When choosing a centrifuge for a specific

process, it is important to ensure that the centrifuge will be able to perform the separation at the planned production rate, and operate reliably with minimum manpower. Large-scale tests may therefore be necessary with fermentation broths or other materials to check that the correct centrifuge is chosen.

10.8.2 BASKET CENTRIFUGE (PERFORATED-BOWL BASKET CENTRIFUGE)

Basket centrifuges are useful for separating mould mycelia or crystalline compounds. The centrifuge is most commonly used with a perforated bowl lined with a filter bag of nylon, cotton, etc. A continuous feed is used, and when the basket is filled with the filter cake, it is possible to wash the cake before removing it. The bowl may suffer from blinding with soft biological materials so that high centrifugal forces cannot be used. These centrifuges are normally operated at speeds of up to 4000 rpm for feed rates of 50–300 dm3 min–1 and have a solids holding capacity of 30–500 dm3. The basket centrifuge may be considered to be a Centrifugal filter.

10.8.3 TUBULAR-BOWL CENTRIFUGE

This is a centrifuge to consider using for particle size ranges of 0.1–200 µm and up to 10% solids in the in-going slurry and arrangement used in a Sharples Super Centrifuge. The main component of the centrifuge is a cylindrical bowl (or rotor), which may be of a variable design depending on application, suspended by a flexible shaft, driven by an overhead motor or air turbine . The inlet to the bowl is via a nozzle attached to the bottom bearing. The feed which may consist of solids and light and heavy liquid phases is introduced by the nozzle. During operation solids sediment on the bowl wall while the liquids separate into the heavy phase in zone and the light phase in the central zone. The two liquid phases are kept separate in their exit from the bowl by an adjust- able ring, with the heavy phase flowing over the lip of the ring. Rings of various sizes may be fitted for the separation of liquids of various relative densities. Thus the centrifuge may be altered to use for:

1. Light-phase/heavy-phase liquid separation.
2. Solids/light-liquid phase/heavy-liquid phase separation.
3. Solids/liquid separation

The Sharpies laboratory centrifuge with a bowl radius of approximately 2.25 cm can be operated with an air turbine at 50,000 rpm to produce a centrifugal force of approximately 62,000g, but has a

bowl capacity of only 200 cm3 with a throughput of 6–25 dm3 h–1. The largest size rotor is the Sharpies AS 26, which has a bowl radius of 5.5 cm and a capacity of 9 dm3, a solids capacity of 5 dm3 and a throughput of 390–2400 dm3 h–1. The advantages of this design of centrifuge are the high centrifugal force, good dewatering, and ease of cleaning. The disadvantages are limited solids capacity, difficulties in the recovery of collected solids, gradual loss in efficiency as the bowl fills, solids being dislodged from the walls as the bowl is slowing down, and foaming. Plastic liners can be used in the bowls to help improve batch cycle time. Alternatively a spare bowl can be changed over in about 5 min

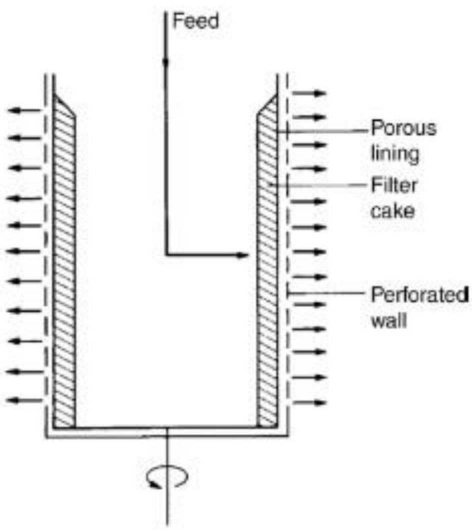


Fig 8. Diagram of Basket Centrifuge turbine

10.8.4 THE SOLID-BOWL SCROLL CENTRIFUGE (DECANTER CENTRIFUGE)

This type of centrifuge is used for continuous handling of fermentation broths, cell lysates and coarse materials such as sewage sludge. The slurry is fed through the spindle of an Archimedean screw within the horizontal rotating solids bowl. Typically the speed differential between the bowl and the screw is in the range 0.5–100 rpm. The solids settling on the walls of the bowl are scraped to the conical end of the bowl. The slope of the cone helps to remove excess liquid from the solids before discharge. The liquid phase is dis- charged from the opposite end of the bowl. The speed of this type of centrifuge is limited to around 5000 rpm in larger models because of the lack of balance within the bowl, with smaller models having bowl speeds of up to

10000 rpm. Bowl diam- eters are normally between 0.2 and 1.5 m, with the length being up to 5 times the diameter. Feed rates range from around 200 dm3 h–1 to 200 m3 h–1 depending on scale of operation and material being processed.

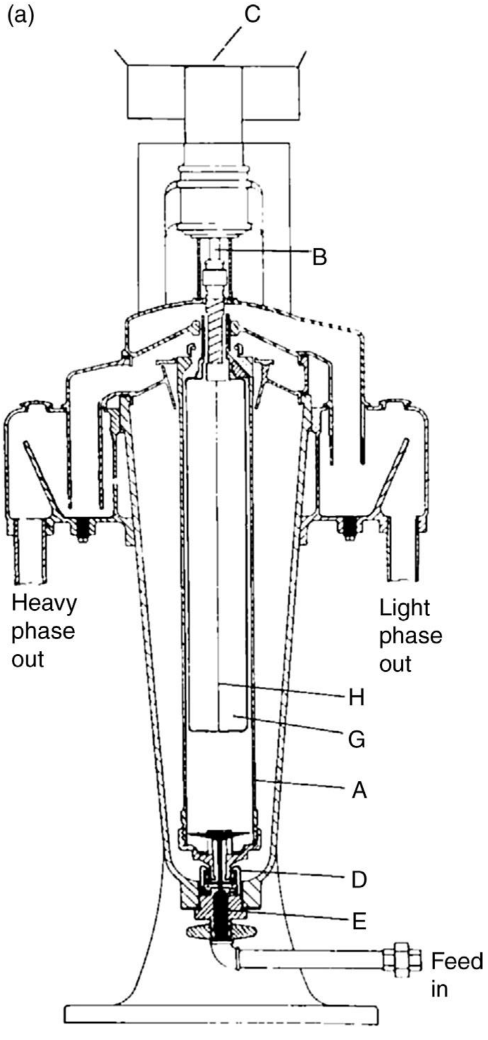


Figure-9: Tubular-bowl centrifuge. a)Section of a Sharples Stiper-Centrifuge (Alfa Laval Sharpies, Camberley, UK). (b) A Sharples Super-Centrifuge assembled for discharge of one liquid phase (Alfa Laval Sharpies, Camberley, UK)

10.8.5 MULTICHAMBER CENTRIFUGE

Ideally, this is a centrifuge for slurry of up to 5% solids of particle size 0.1–200 µm diameter. In the multichamber centrifuge a series of concentric cham- bers are mounted within the rotor chamber. The broth enters via the central spindle and then takes a circuitous route through the chambers. Solids collect on the outer faces of each

chamber. The smaller particles collect in the outer chambers where they are subjected to greater centrifugal forces (the greater the radial position of a particle, the greater the rate of sedimentation). Although these vessels can have a greater solids capacity than tubular bowls and there is no loss of efficiency as the chamber fills with solids, their mechanical strength and design limits their speed to a maximum of 6500 rpm for a rotor 46-cm diameter with a holding capacity of up to 76 dm3. Because of the time needed to dismantle and recover the solids fraction, the size and number of vessels must be of the correct volume for the solids of a batch run.

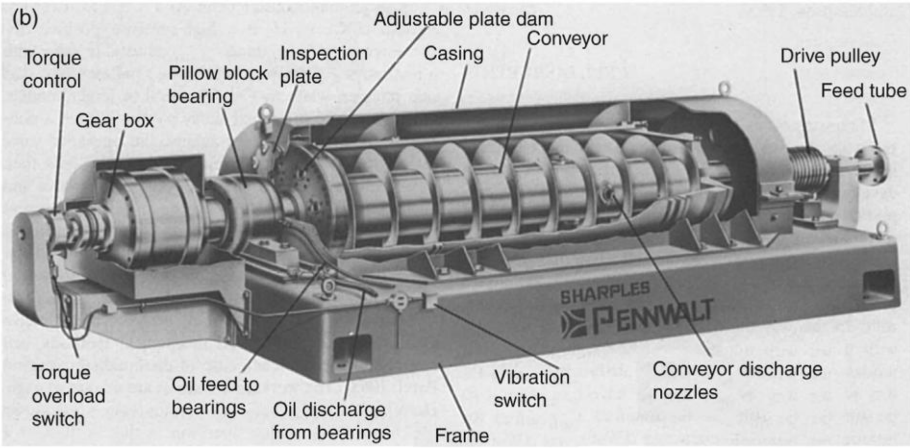


Figure-10. Diagram of a solid-bowl scroll centrifuge (Alfa Laval Sharpies Ltd, Camberley, UK). (b) Cutaway view of a Sharpies Super-D-Canter continuous solid-bowl centrifuge, Model P-5400 (Alfa Laval Sharpies Ltd, Camberley, UK).

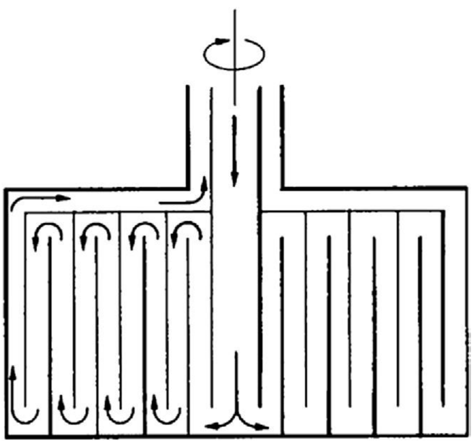


Figure -11. L.S. of a Multichamber Centrifuge

10.8.6 DISC-BOWL CENTRIFUGE

This centrifuge relies for its efficiency on the presence of discs in the rotor or bowl (Fig. 12). A central inlet pipe is surrounded by a stack of stainless-steel conical discs. Each disc has spacers so that a stack can be built up. The broth to be separated flows outward from the central feed pipe, then upward and inward between the discs at an angle of 45 degrees to the axis of rotation. The close packing of the discs assists rapid sedimentation and the solids then slide to the edge of the bowl, provided that there are no gums or fats in the slurry, and eventually accumulate on the inner wall of the bowl. Ideally, the sediment should form a sludge which flows, rather than a hard particulate or lumpy sediment. The main advantages of these centrifuges are their small size compared with a bowl without discs for a given throughput. Some designs also have the facility for continuous solids removal through a series of nozzles in the circumference of the bowl or intermittent solids removal by automatic opening of the solids collection bowl. The arrangement of the discs makes this type of centrifuge laborious to clean. However, recent models such as the Alfa Laval BTUX 510 (Alfa Laval Sharpies Ltd, Camberley, Surrey, U.K.) system (Fig. 12) are designed to allow for cleaning in situ. In addition, this and similar plant have the facility for in situ steam sterilization and total containment, incorporating double seals to comply with containment regulations. Feed rates range from 45 to 1800 dm3 min–1, with rotational speeds typically between 5000 and 10,000 rpm. The Westfalia CSA 19–47–476 is also steam sterilizable and has been used for the sterile collection of organisms.

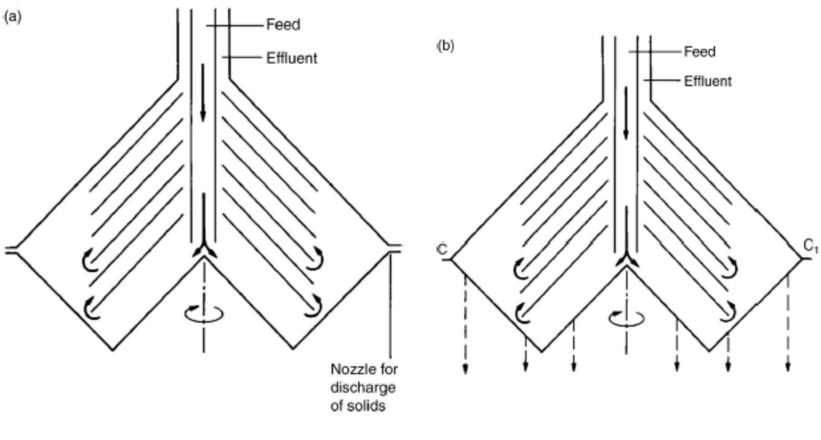


Figure – 12. (a) L.S. of disc-bowl centrifuge with nozzle discharge. (b) L.S. of disc-bowl centrifuge with intermittent discharge. (Solids discharged when rotor opens intermittently along the section C–Cj.)

NOTES

Check your Progress -1

Note: Write your answer in the space given below

a. Name any two antifoam agents.

b. Any two centrifuges for cell harvesting.

………………………………………………………

……………………..

10.9 LET US SUM UP

In this unit, you have learnt about the basic techniques of the filters and centrifuges for large scale purification of fermented products.

10.10 UNIT - END EXERCISES

1. List out the importance of filters.
2. List out the centrifuges used for industrial use.

10.11 ANSWERS TO CHECK YOUR PROGRESS

1. Amonium sulphate, mineral oil.
2. Disc Stack Centrifuge, centrifuge, membrane centrifuge.

10.12 SUGGESTED READINGS

2. Otero, J. M., & Nielsen, J. (2010). Industrial systems biology. *Biotechnology and Bioengineering*, *105*(3), 439–460.
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UNIT -XI: DOWNSTREAM PROCESSING - III

Structure

11.1 INTRODUCTION

In this chapter, deals with the methods of purification of industrially valuable products, especially intracellular metabolites. The first step of the process is cell lysis, methods of cell disruption, which includes physical nd chemical methods. Further, extraction methods such as chromatography, membrane process, drying and whole broth processing also discussed in this chapter.

11.2 OBJECTIVES

After going through the unit you will be able to;

- Understand cell lysis methods.
- Know the physical and chemical methods of cell lysis.
- Know the various extraction methods.
- Understand the chromatography principles.
- Value the role membrane process for purification of the product.
- Know the methods of crystallization and drying the product.
- To know the method of whole broth processing.

11. 3 CELL DISRUPTION

Microorganisms are protected by extremely tough cell walls. In order to release their cellular contents a number of methods for cell disintegration have been developed. Any potential method of disruption must ensure that labile materials are not denatured by the process or hydrolyzed by enzymes present in the cell. The use of a combination of different techniques to release products from specific locations within yeast cells. In this way the desired product can be obtained with minimum contamination. Although many techniques are available which are satisfactory at laboratory scale, only a limited number have been proved to be suitable for large-scale applications, particularly for intracellular enzyme extraction. Containment of cells can be difficult or costly to achieve in many of the methods described later and thus containment requirements will strongly influence process choice. Methods available fall into two major categories

Physico mechanical methods

1. Liquid shear. 2. Solid shear. 3. Agitation with abrasives. 4. Freeze thawing. 5. Ultrasonication. 6. Hydrodynamic cavitations.

Chemical and biological methods

1. Detergents. 2. Osmotic shock. 3. Alkali treatment. 4. Enzyme treatment. 5. Solvents.

11. 4 PHYSICAL AND CHEMICAL METHOD

11.4.1 PHYSICAL METHODS

LIQUID SHEAR (HIGH-PRESSURE HOMOGENIZERS)

Liquid shear is the method which has been most widely used in large scale enzyme purification procedures. High-pressure homogenizers used in the processing of milk and other products in the food industry have proved to be very effective for microbial cell disruption. One machine, the APV-Manton Gaulin-homogenizer (The APV Co. Ltd, Crawley, Surrey, UK), which is a high-pressure positive displacement pump, incorporates an adjustable valve with a restricted orifice. The smallest model has one plunger, while there are several in larger models. During use, the microbial slurry passes through a nonreturn valve and impinges against the operative valve set at the

selected operating pressure. The cells then pass through a narrow channel between the valve and an impact ring followed by a sudden pressure drop at the exit to the narrow orifice. There are various discharge valve designs with sharp edged orifices being preferred for cell disruption. The large pressure drop across the valve is believed to cause cavitations in the slurry and the shock waves so produced disrupt the cells. The size of the pressure drop to be very important in achieving effective disruption, and as with all mechanical methods, cell size and shape influence ease of disruption.

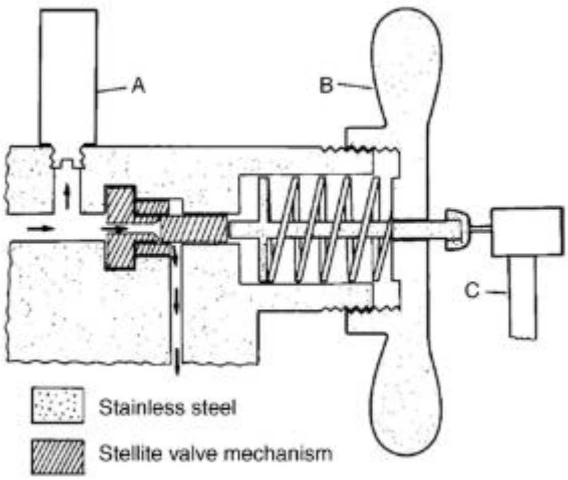


Fig. 1 Details of Homogonizer Valve Assembly (Brookman, 1974)
Solid shear

Pressure extrusion of frozen microorganisms at around −25°C through a small ori- fice is a well established technique at a laboratory scale using a Hughes press or an X-press to obtain small samples of enzymes or microbial cell walls. Disruption is due to a combination of liquid shear through a narrow orifice and the presence of ice crystals and developed a semicontinuous X-press operating with a sample temperature of −35°C and an X-press temperature of −20°C. It was possible to obtain 90% disruption with a single passage of S. cereuisiae using a throughput of 10 kg yeast cell paste h–1. This technique might be ideal for microbial products which are very temperature labile. Agitation with abrasives (high speed bead mills).

Mechanical cell disruption can also be achieved in a disintegrator containing a series of rotating discs/impellers on a central drive shaft and a charge of small beads. Beads are typically 0.1−3 mm

diameter depending on the type of microorganism and impeller tip speeds are in the order of 15 m s–1. The beads are made of mechanically resistant materials such as glass, alumina ceramics and some titanium compounds. Disruption is achieved through interparticle collision and solid shear. In a small disintegrator, the Dyno-Muhle KD5 using a flow rate of 180 dm3 h–1 , 85% disintegration of an 11% w/v suspension of S. cereuisiae was achieved with a single pass (Fig.2). Although temperatures of up to 35°C were recorded in the disintegrator, the specific enzyme activities were not considered to be very different from values obtained by other techniques. Dissipation of heat generated in the mill is one of the major problems in scale up, though this can generally be overcome with the pro- vision of a cooling jacket. In another disintegrator, the Netzsch LM20 mill (Netzsch GmbH, Selb, Germany), the agitator blades were alternately mounted vertically and obliquely on the horizontal shaft. A flow rate of up to 400 dm3 h–1 was claimed for a vessel with a nominal capacity of 20 dm^3.

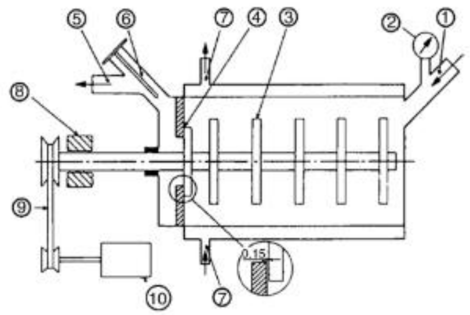


Fig. 2 Simplified Drawing of the Dyno-Muhle KD5 (Mogren et al., 1974)

FREEZING–THAWING

Freezing and thawing of a microbial cell paste will inevitably cause ice crystals to form and their expansion followed by thawing will lead to some subsequent disrup- tion of cells. It is slow, with limited release of cellular materials, and has not often been used as a technique on its own, although it is often used in combination with other techniques. β-Glucosidase has been obtained from S. cerevisiae. A sample of 360 g of frozen yeast paste was thawed at 5°C for 10 h. This cycle was repeated twice before further processing.

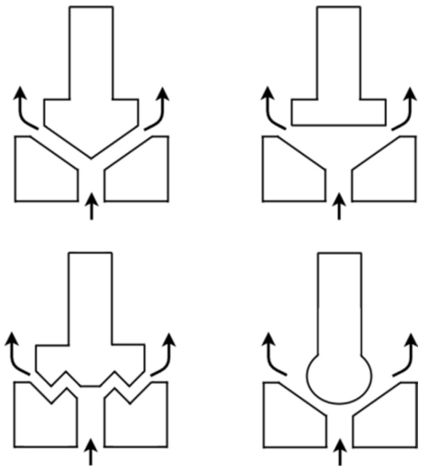


Figure- 3: Discharge Valve Designs in High-Pressure Homogenizers

ULTRASONICATION (ULTRASONIC CAVITATIONS)

High frequency vibration (~20 kHz) at the tip of an ultrasonication probe leads to cavitation (the formation of vapor cavities in low pressure regions), and shock waves generated when the cavities collapse cause cell disruption. The method can be very effective on a small scale (5–500 mL), but a number of serious drawbacks make it unsuitable for large-scale operations. Power requirements are high, there is a large heating effect so cooling is needed, the probes have a short working life and are only effective over a short range. Continuous laboratory sonicators with hold-up volumes of around 10 cm3 have been shown to be effective.

Hydrodynamic cavitations

Cavitation similar to that generated by ultrasonication probes can also be generated by fluid flow. When fluid flows through an orifice an increase in velocity is accompanied by a decrease in pressure of the fluid. When the pressure falls to the vapor pressure of the fluid cavitation occurs resulting in cell damage/disruption.

11.4.2 CHEMICAL AND BIOLOGICAL METHODS

DETERGENTS

A number of detergents will damage the lipoproteins of the microbial cell mem- brane and lead to the release of intracellular components. The compounds which can be used for this purpose include quaternary ammonium compounds, sodium lauryl sulfate, sodium dodecyl sulfate

(SDS) and Triton X-100. Anionic detergents such as SDS disorganize the cell membrane while cationic detergents are believed to act on lipopolysaccharides and phospholipids of the membrane. Nonionic detergents such as Triton X-100 cause partial solubilization of membrane proteins. Unfortunately, the detergents may cause some protein denaturation and may need to be removed before further purification stages can be undertaken. The stability of the desired product must be determined when using any detergent system. Pullulanase is an enzyme which is bound to the outer membrane of Klebsiella pneumoniae. The cells were suspended in pH 7.8 buffer and 1% sodium cholate was added. The mixture was stirred for 1 h to solubilize most of the enzyme (Kroner, Hustedt, Granda, & Kula, 1978). The use of Triton X-100 in combination with guanidine-HCl is widely and effectively used for the release of cellular protein.

OSMOTIC SHOCK

Osmotic shock caused by a sudden change in salt concentration will cause disruption of a number of cell types. Cells are equilibrated to high osmotic pressure (typically 1 M salt solutions). Rapid exposure to low osmotic pressure causes water to quickly enter the cell. This increases the internal pressure of the cell resulting in cell lysis. Osmotic shock is of limited application except where the cell wall is weakened or absent. Application on a large scale is limited by the cost of chemicals, increased water use, and possible product dilution.

ALKALI TREATMENT

Alkali treatment might be used for hydrolysis of microbial cell wall material provid- ed that the desired product will tolerate a pH of 10.5–12.5 for up to 30 min. Chemical costs can be high both in terms of alkali required and neutralization of the resulting lysate.

ENZYME TREATMENT

There are a number of enzymes which hydrolyze specific bonds in cell walls of a limited number of microorganisms. Enzymes shown to have this activity include lysozyme, produced from hen egg whites and other natural sources, and other enzyme extracts from leucocytes, Streptomyces spp., Staphylococcus spp., Micromonospora spp. Penicillium spp., Trichoderma spp., and snails. Lysozyme hydrolyses β-1-4 glucosidic bonds in the polysaccharide chains of peptidoglycan causing cell lysis. Although this is probably one of the most gentle

methods available, unfortunately it is relatively expensive and the presence of the enzyme(s) may complicate further downstream purification processes. Enzyme lysis in large scale operations is limited by the availability and cost of appropriate enzymes. The use of immobilized lyso- zyme has been investigated by a number of workers and may provide the solution to such problems.

SOLVENTS

Solvents extract the lipid components of the cell membrane causing the release of intracellular components and are applicable across a wide range of microorganisms. Solvents used include alcohols, dimethyl sulfoxide, methyl ethyl ketone, and tolu- ene. However, their toxicity, flammability, and ability to cause protein denaturation requires careful consideration. Chemical and enzymatic methods for the release of intracellular products have not been used widely on a large scale, with the exception of lysozyme. However, their potential for the selective release of product and that they often yield a cleaner lysate mean that they are potentially invaluable tools in the recovery of fermentation products. Enzymes may also be used as a pretreatment to partially hydrolyze cell walls prior to cell disruption by mechanical methods.

11.5 LIQUID LIQUID EXTRACTION

The separation of a component from a liquid mixture by treatment with a solvent in which the desired component is preferentially soluble is known as liquid–liquid extraction. The specific requirement is that a high percentage extraction of product must be obtained but concentrated in a smaller volume of solvent. Prior to starting a large-scale extraction, it is important to find out on a small scale the solubility characteristics of the product using a wide range of solvents. A simple rule to remember is that “like dissolves like.” The important “likeness” as far as solubility relations are concerned is in the polarities of molecules. Polar liquids mix with each other and dissolve salts and other polar solids. The solvents for nonpolar compounds are liquids of low or nil polarity. The dielectric constant is a measure of the degree of molar polarization of a com- pound. If this value is known it is then possible to predict whether a compound will be polar or nonpolar, with a high value indicating a highly polar compound. The dielectric constant D of a substance can be measured by determining the

NOTES

electrostatic capacity C of a condenser containing the substance between the plates.

If C0 is the value for the same condenser when completely evacuated then

$$D = \frac{C}{C0}$$

Experimentally, dielectric constants are obtained by comparing the capacity of the condenser when filled with a given liquid with the capacity of the same condenser containing a standard liquid whose dielectric constant is known very accurately. If D1 and D2 are the dielectric constants of the experimental and standard liquids and C1 and C2 are the electrostatic capacities of a condenser when filled with each of the liquids, then

$$\frac{D1}{D2} = \frac{C1}{C2}$$

The value of D1 can be calculated since C1 and C2 can be measured and D2 is known. The dielectric constants for a number of solvents are given in table 1. The final choice of solvent will be influenced by the distribution or partition coefficient K where

$$K = \frac{\text{Concentration in solute extract}}{\text{Concentration of solute in raffinate}}$$

Table 1. Dielectric Constants of Solvents at 25°C (Arranged in Order of Increasing Polarity).

Solvent	Dielectric Constant
Hexane	1.90 (least polar
Cyclohexane	2.02
Carbon tetrachloride	2.24
Benzene	2.28
Di-ethyl ether	4.34
Chloroform	4.87
Ethyl acetate	6.02
Butan-2-ol	15.8
Butan-l-ol	17.8
Propan-l-ol	20.1
Acetone	20.7
Ethanol	24.3
Methanol	32.6
Water	78.5 (most polar)

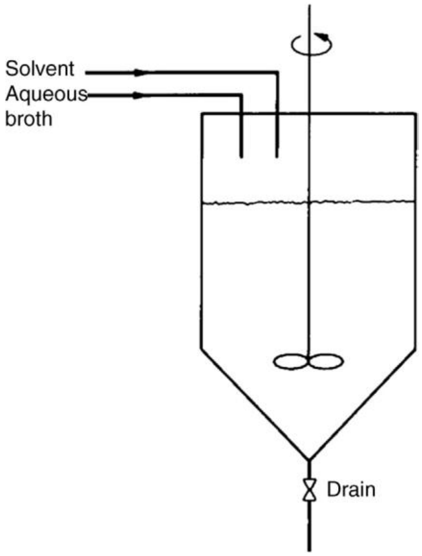


Figure-4: Diagram of a Single-Stage Extraction Unit.

The value of *K* defines the ease of extraction. When there is a relatively high *K* value, good stability of product and good separation of the aqueous and solvent phases, then it may be possible to use a single-stage extraction system (Fig. 4). A value of 50 indicates that the extraction should be straightforward whereas a value of 0.1 shows that the extraction will be difficult and that a multistage process will be necessary. Unfortunately, in a number of systems the value of *K* is low and cocurrent or countercurrent multistage systems have to be utilized.

The cocurrent system is illustrated in Fig. 5. There are *n* mixer/separator vessels in line and the raffinate goes from vessel 1 to vessel *n*. Fresh solvent is added to each stage, the feed and extracting solvent pass through the cascade in the *same* direction. Extract is recovered from each stage. Although a relatively large amount of solvent is used, a high degree of extraction is achieved.

A countercurrent system is illustrated in Fig. 6. There are a number of mixer/separators connected in series. The extracted raffinate passes from vessel 1 to vessel *n* while the product-enriched solvent is flowing from vessel *n* to vessel 1. The feed and extracting solvent pass through the cascade in *opposite* directions. The most efficient system for solvent utilization is countercurrent operation, showing a considerable advantage over batch and cocurrent systems. Unless there are special reasons the counter-current system should be used. In practice, the series of countercurrent extractions are conducted in a single continuous extractor using centrifugal forces to separate the two liquid

phases. The two liquid streams are forced to flow countercurrent to each other through a long spiral of channels within the rotor.

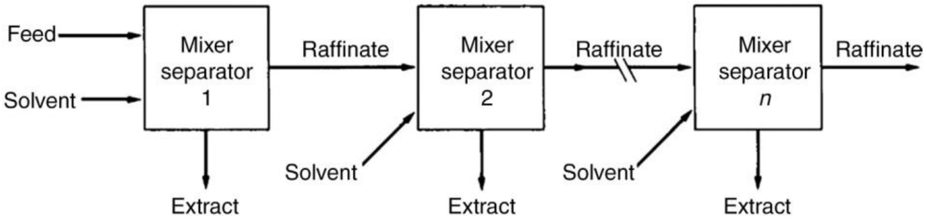


Figure- 5: Diagram of a Cocurrent Flow Extraction System

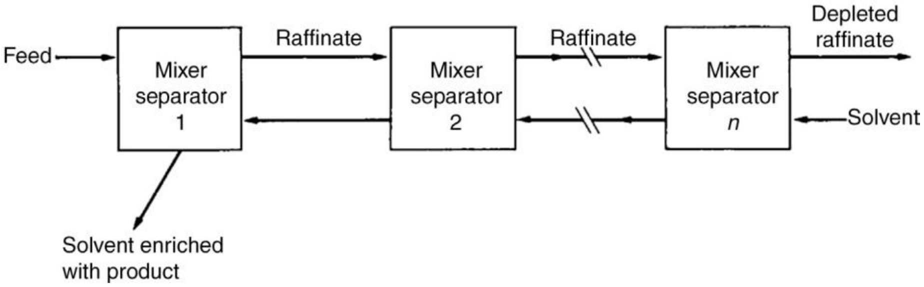


Figure-6: Diagram of a Countercurrent Extraction System

11.6 SOLVENT RECOVERY

A major item of equipment in an extraction process is the solvent-recovery plant which is usually a distillation unit. It is not normally essential to remove all the raffinate from the solvent as this will be recycled through the system. In some processes the more difficult problem will be to remove all the solvent from the raffinate because of the value of the solvent and problems which might arise from contamination of the product.

Distillation may be achieved in three stages:

1. Evaporation, the removal of solvent as a vapor from a solution.
2. Vapor–liquid separation in a column, to separate the lower boiling more volatile component from other less volatile components.
3. Condensation of the vapor, to recover the more volatile solvent fraction.

Evaporation is the removal of solvent from a solution by the application of heat to the solution. A wide range of evaporators is available. Some are operated on a batch basis and others continuously. Most industrial evaporators employ tubular heating surfaces. Circulation of the liquid past the heating surfaces may be induced by boiling or by mechanical

agitation. In batch distillation (Fig.7), the vapor from the boiler passes up the column and is condensed. Part of the condensate will be returned as the reflux for countercurrent contact with the rising vapor in the column. The distillation is continued until a satisfactory recovery of the lower-boiling (more volatile) component(s) has been accomplished. The ratio of condensate returned to the column as reflux to that withdrawn as product is, along with the number of plates or stages in the column, the major method of controlling the product purity.

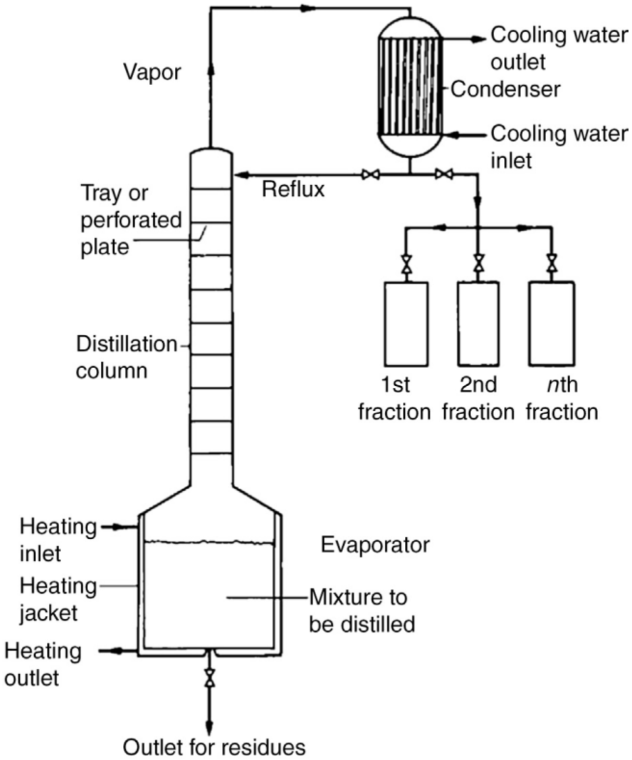


Figure-7: Diagram of a Batch Distillation Plant With a Tray or Perforated-Plate Column.

A continuous distillation is (Fig.8) initially begun in a similar way as with a batch distillation, but no condensate is withdrawn initially. There is total reflux of the condensate until ideal operating conditions have been established throughout the column. At this stage the liquid feed is fed into the column at an intermediate level. The more volatile components move upward as vapor and are condensed, followed by partial reflux of the condensate. Meanwhile, the less volatile fractions move down the column to the evaporator (reboiler). At this stage part of the bottoms fraction is continuously with- drawn and part is reboiled and returned to the column.

The plate or tray column consists of a number of distinct chambers (Fig.7) separated by perforated plates or trays. The rising vapor bubbles through the liquid which is flowing across each plate, and is dispersed into the liquid from perforations (sieve plates) or bubble caps. The liquid flows across the plates and reaches the reboiler by a series of overflow wires and down pipes.

A packed tower is filled with a randomly packed material such as rings, saddles, helices, spheres or beads. Their dimensions are approximately one-tenth to one-fiftieth of the diameter of the column and are designed to provide a large surface area for liquid-vapor contacting and high voidage to allow high throughput of liquid and vapor.

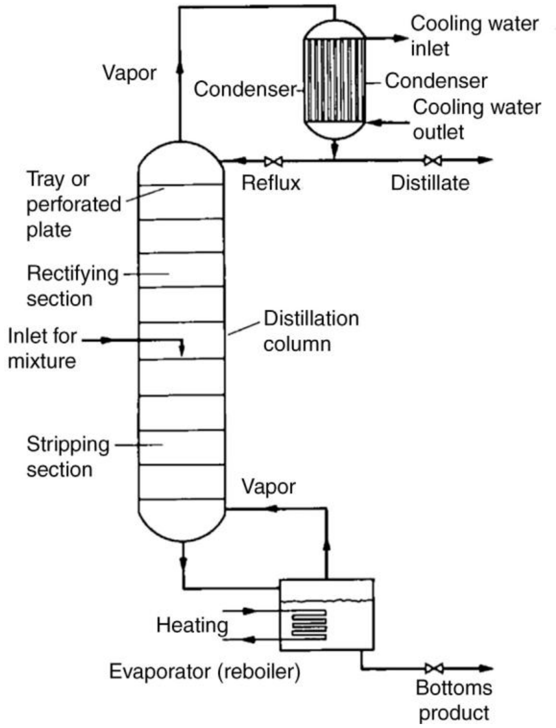


Figure-8: of a Continuous Distillation Plant With a Tray or Perforated-Plate Column.

The heat input to a distillation column can be considerable. The simplest ways of conserving heat are to preheat the initial feed by a heat exchanger using heat from:

1. The hot vapors at the top of the column,

2. Heat from the bottoms fraction when it is being removed in a continuous process,

3. A combination of both.

11.7 TWO-PHASE AQUEOUS EXTRACTION

Liquid–liquid extraction is a well established technology in chemical processing and in certain sectors of biochemical processing. However, the use of organic solvents has limited application in the processing of sensitive biologicals. Aqueous two-phase systems, on the other hand, have a high water content and low interfacial surface tension and are regarded as being biocompatible. Two-phase aqueous systems have been known since the late 19th century, and a large variety of natural and synthetic hydrophilic polymers are used today to create two (or more) aqueous phases. Phase separation occurs when hydrophilic polymers are added to an aqueous solution, and when the concentrations exceed a certain value two immiscible aqueous phases are formed. Settling time for the two phases can be pro- longed, depending on the components used and vessel geometry. Phase separation can be improved by using centrifugal separators, or novel techniques such as magnetic separators.

Many systems are available:

1. Nonionic polymer/nonionic polymer/water, for example, polyethylene glycol/ dextran.
2. Polyelectrolyte/nonionic polymer/water, for example, sodium carboxymethyl cellulose/polyethylene glycol.
3. Polyelectrolyte/polyelectrolyte/water, for example, sodium dextran sulfate/ sodium carboxymethyl cellulose.
4. Polymer/low molecular weight component/water, for example, dextran/propyl alcohol.

The distribution of a solute species between the phases is characterized by the partition coefficient, and is influenced by a number of factors such as temperature, polymer (type and molecular weight), salt concentration, ionic strength, pH, and properties (eg, molecular weight) of the solute. As the goal of any extraction process is to selectively recover and concentrate a solute, affinity techniques such as those applied in chromatographic processes can be used to improve selectivity. Examples include the use of PEG–NADH derivatives in the extraction of dehydrogenases, p-aminobenzamidine in the extraction of trypsin and cibacron blue in the extrac- tion of phosphofructokinase. It is possible to use different ligands in the two phases leading to an increase in selectivity or the simultaneous recovery and separation of several species. A comparison of aqueous two-phase separation and ion-exchange chromatography shows that the process yield and costs

are lower for the aqueous two-phase process. Two phase aqueous systems have found application in the purification of many sol- utes; proteins, enzymes recombinant proteins using a PEG/salt system β-carotenene and lutein from cyanobacterial fermentations utilizing a PEG/salt process, antibiotics, cells and subcellular particles, and in extractive bioconversions. The cost of phase forming polymers and chemicals have limited the use of aqueous two-phase processes in industrial applications, however some aqueous two-phase systems for handling large-scale protein separation have emerged, the majority of which use PEG as the upper phase forming polymer with either dextran, concentrated salt solution or hydroxypropyl starch as the lower phase forming material. The demonstrated the application of continuous cross-current extraction of enzymes (fumarase and penicillin acylase) by aqueous two-phase systems at production scale.

11.7.1 REVERSED MICELLE EXTRACTION

Reversed micelle extraction is potentially an attractive alternative to conventional solvent extraction for the recovery of bioproducts as the solute of interest remains in an aqueous environment at all times and hence can be considered "biocompat- ible." A reversed micelle is a nanometer scale droplet of aqueous solution stabilized in a nonpolar environment by a surfactant at the interface between the two liquids. The minimum concentration of surfactant required for micelles is known as the critical micelle concentration (CMC) and is highly system specific. There are a number of limitations to reversed micelle extraction including costs and low rates of mass transfer.

11.7.2 SUPERCRITICAL FLUID EXTRACTION

The technique of supercritical fluid extraction utilizes the dissolution power of super- critical fluids, that is, fluids above their critical temperature and pressure. Its advantages include the use of moderate temperatures, good solvent, and transport properties (high diffusivity and low viscosity), and that cheap and nontoxic fluids are available. Supercritical fluids are used in the extraction of hop oils, caffeine, vanilla, vegetable oils, and β-carotene. It has also been shown experimentally that the extraction of certain steroids and chemotherapeutic drugs can be achieved using supercritical fluids. Other current and potential uses include the removal of undesirable substances such as pesticide residues, removal of bacteriostatic agents from fermentation broths, the recovery of organic solvents from

aqueous solutions, cell disintegration, destruction and treatment of industrial wastes, and liposome preparation. Supercritical fluids are used in the extraction of hop oils, caffeine, vanilla, veg- etable oils, and β-carotene. It has also been shown experimentally that the extraction of certain steroids and chemotherapeutic drugs can be achieved using supercritical fluids. Other current and potential uses include the removal of undesirable substances such as pesticide residues, removal of bacteriostatic agents from fermentation broths, the recovery of organic solvents from aqueous solutions, cell disintegration, destruction and treatment of industrial wastes, and liposome preparation. There are, how- ever, a number of significant disadvantages in the utilization of this technology:

1. Phase equilibria of the solvent/solute system is complex, making design of extraction conditions difficult.
2. The most popular solvent (carbon dioxide) is nonpolar and is therefore most useful in the extraction of nonpolar solutes. Though cosolvents can be added for the extraction of polar compounds, they will complicate further downstream processing.
3. The use of high pressures leads to high capital costs for plant, and operating costs may also be high.

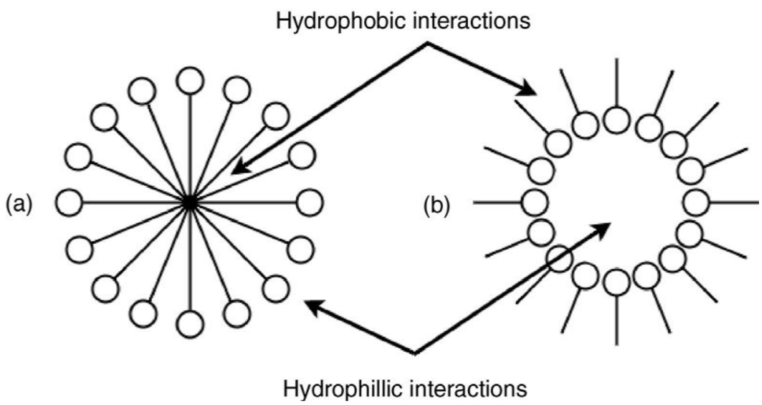


Figure-9: Micelles. a) Normal; b) reversed.

Thus, the number of commercial processes utilizing supercritical fluid extraction is relatively small, due mainly to the existence of more economical processes. How- ever, its use is increasing, for example, the recovery of high value biologicals, when conventional extractions are inappropriate, and in the treatment of toxic wastes. Super critical CO2 extraction has been described for the extraction of 2-phenylethyl alcohol (rose aroma) from cell free extracts of Kluyveromyces marxianus fermentations at pressures of 200 bar and

temperatures between 35 and 45oC. The distribution coefficient was found to be twice that of conventional solvent extraction using n-hexane with greater than 90% product extraction.

11. 8 CHROMATOGRAPHY

In many fermentation processes, chromatographic techniques are used to isolate and purify relatively low concentrations of metabolic products. Chromatographic methods separate solutes based on charge, polarity, size, and affinity. In this context, chromatography will be concerned with the passage and separation of different solutes as liquid (the mobile phase) is passed through a column, that is, liquid chromatography. Gas chromatography, when the mobile phase is a gas, is a widely used analytical technique but has little application in the recovery of fermentation products. Depend- ing on the mechanism by which the solutes may be differentially held in a column, the techniques can be grouped as follows:

1. Adsorption chromatography.
2. Ion-exchange chromatography.
3. Gel permeation chromatography.
4. Affinity chromatography.
5. Reverse phase chromatography.
6. High performance liquid chromatography.

Chromatographic techniques are also used in the final stages of purification of a number of products. The scale-up of chromatographic processes can prove difficult, mainly as a result of the pressures used causing compaction of the column packing materials, and there is much current interest in the use of mathematical models and computer programs to translate data obtained from small-scale processes into operating conditions for larger scale applications.

11.8.1 ADSORPTION CHROMATOGRAPHY

Adsorption chromatography involves binding of the solute to the solid phase primar- ily by weak Van de Waals forces. The materials used for this purpose to pack columns include inorganic adsorbents (active carbon, aluminum oxide, aluminum hydroxide, magnesium oxide, silica gel) and organic macroporous resins. Adsorption and affinity chromatography are mechanistically identical, but are strategically different. In affinity systems selectivity is designed rationally while in

adsorption selectivity must be determined empirically. Dihydrostreptomycin can be extracted from filtrates using activated charcoal columns. It is then eluted with methanolic hydrochloric acid and purified in further stages. Some other applications for small-scale antibiotic purification are quoted. Active carbon may be used to remove pigments to clarify broths. Penicillin- containing solvents may be treated with 0.25–0.5% active carbon to remove pigments and other impurities.

11.8.2 ION EXCHANGE

Ion exchange can be defined as the reversible exchange of ions between a liquid phase and a solid phase (ion-exchange resin) which is not accompanied by any radical change in the solid structure. Cationic ion-exchange resins normally contain a sulfonic acid, carboxylic acid, or phosphonic acid active group. Carboxy-methyl cellulose is a common cation exchange resin. Positively charged solutes (eg, certain proteins) will bind to the resin, the strength of attachment depending on the net charge of the solute at the pH of the column feed. After deposition solutes are sequentially washed off by the passage of buffers of increasing ionic strength or pH. Anionic ion-exchange resins normally contain a secondary amine, quaternary amine, or quaternary ammonium active group. A common anion exchange resin, DEAE (diethylaminoethyl) cellulose is used in a similar manner to that described earlier for the separation of negatively charged solutes. Other functional groups may also be attached to the resin skeleton to provide more selective behavior similar to that of affinity chromatography. The appropriate resin for a particular purpose will depend on various factors such as bead size, pore size, diffusion rate, resin capacity, range of reactive groups, and the life of the resin before replacement is necessary. Weak-acid cation ion-exchange resins can be used in the isolation and purification of streptomycin, neomycin, and similar antibiotics

18.8.3 GEL PERMEATION

This technique is also known as gel exclusion and gel filtration. Gel permeation separates molecules on the basis of their size. The smaller molecules diffuse into the gel more rapidly than the larger ones, and penetrate the pores of the gel to a greater degree. This means that once elution is started, the larger molecules which are still in the voids in the gel will be eluted first. A wide range of gels are available, including

crosslinked dextrans (Sephadex and Sephacryl) and crosslinked agarose (Sepharose) with various pore sizes depending on the fractionation range required. One early industrial application, although on a relatively small scale, was the purification of vaccines. Tetanus and diphtheria broths for batches of up to 100,000 human doses are passed through a 13 dm3 column of G 100 followed by a 13 dm3 column of G 200. This technique yields a fairly pure fraction which is then concentrated 10-fold by pressure dialysis to remove the eluent buffer (Na2 HPO4).

18.8.4 AFFINITY CHROMATOGRAPHY

Affinity chromatography is a separation technique with many applications since it is possible to use it for separation and purification of most biological molecules on the basis of their function or chemical structure. This technique depends on the highly specific interactions between pairs of biological materials such as enzyme–substrate, enzyme–inhibitor, antigen–antibody, etc. The molecule to be purified is specifically adsorbed from, for example, a cell lysate applied to the affinity column by a binding substance (ligand) which is immobilized on an insoluble support (matrix). Eluent is then passed through the column to release the highly purified and concentrated molecule. The ligand is attached to the matrix by physical absorption or chemically by a covalent bond. The pore size and ligand location must be carefully matched to the size of the product for effective separation. The latter method is preferred whenever possible.

Coupling procedures have been developed using cyanogen bromide, bisoxiranes, disaziridines, and perio-dates, for matrixes of gels and beads. Four polymers, which are often used for matrix materials are agarose, cellulose, dextrose, and polyacryl- amide. Agarose activated with cyanogen bromide is one of the most commonly used supports for the coupling of amino ligands. Silica based solid phases have been shown to be an effective alternative to gel supports in affinity chromatography.

Purification may be several 1000-fold with good recovery of active material. The method can however be quite costly and time consuming, and alternative affinity methods such as affinity cross-flow filtration, affinity precipitation, and affinity partitioning may offer some advantages. Affinity chromatography was used initially in protein isolation and purification, particularly enzymes. Since then many other

large-scale applications have been developed for enzyme inhibitors, antibodies, interferon, and recombinant proteins and on a smaller scale for nucleic acids, cell organelles, and whole cells. In the scale-up of affinity chromatographic processes bed height limits the superficial velocity of the liquid, thus scale-up requires an increase in bed diameter or adsorption capacity.

11.8.5 REVERSE PHASE CHROMATOGRAPHY (RPC)

When the stationary phase has greater polarity than the mobile phase it is termed “normal phase chromatography.” When the opposite is the case, it is termed “reverse phase chromatography.” RPC utilizes a solid phase (eg, silica) which is modified so as to replace hydrophilic groups with hydrophobic alkyl chains. This allows the separation of proteins according to their hydrophobicity. More-hydrophobic pro- teins bind most strongly to the stationary phase and are therefore eluted later than less-hydrophobic proteins. The alkyl groupings are normally eight or eighteen car- bons in length (C8 and C18). RPC can also be combined with affinity techniques in the separation of, for example, proteins and peptides.

11.8.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a high resolution column chromatographic technique. Improvements in the nature of column packing materials for a range of chromatographic techniques (eg, gel permeation and ion-exchange) yield smaller, more rigid, and more uniform beads. This allows packing in columns with minimum spaces between the beads, thus minimizing peak broadening of eluted species. It was originally known as high pressure liquid chromatography because of the high pressures required to drive sol- vents through silica based packed beds. Improvements in the performance led to the name change and its widespread use in the separation and purification of a wide range of solute species, including biomolecules. HPLC is distinguished from liq- uid chromatography by the use of improved media (in terms of their selectivity and physical properties) for the solid (stationary) phase through which the mobile (fluid) phase passes.

The stationary phase must have high surface area/unit volume, even size and shape and be resistant to mechanical and chemical damage. However, it is fac- tors such as these which lead to high pressure requirements and cost. This may be acceptable for analytical

work, but not for preparative separations. Thus, in preparative HPLC some resolution is often sacrificed (by the use of larger stationary-phase particles) to reduce operating and capital costs. For very high value products, large- scale HPLC columns containing analytical media have been used. Fast protein liquid chromatography (FPLC) is a variant of HPLC which is more suited to large scale purification processes (Doran, 2013). Affinity techniques can be merged with HPLC to combine the selectivity of the former with the speed and resolving power of the latter.

11.8.7 CONTINUOUS CHROMATOGRAPHY

Although the concept of continuous enzyme isolation is well established, the stage of least development is continuous chromatography (Fig.10). The possible are continuous chromatography alternatives to batch chromatography. These include annular chromatography, carrousel chromatography, and various configurations of moving bed chromatography. The developed a continuous-fed annular column for this purpose. It consisted of two concentric cylindrical sections clamped to a base plate. The space (1 cm wide) between the two sections was packed with the appropriate resin or gel giving a total column capacity of 2.58 dm3 . A series of orifices in the circumference of the base plate below the column space led to collecting ves- sels. The column assembly was rotated in a slow-moving turntable (0.4–2.0 rpm). The mixture for separation was fed to the apparatus by an applicator rotating at the same speed as the column, thus allowing application at a fixed point, while the eluent was fed evenly to the whole circumference of the column. The components of a mixture separated as a series of helical pathways, which varied with the retention proper- ties of the constituent components. This method gave a satisfactory separation and recovery but the consumption of eluent and the unreliable throughput rate were not considered to be satisfactory for a large-scale method.

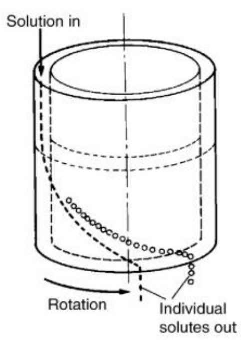


Fig 10. The Principle of Continuous-Partition Chromatography

11.9 MEMBARANE PROCESSING

Both processes utilize semipermeable membranes to separate molecules of different sizes and therefore act in a similar manner to conventional filters.

11.9.1 ULTRAFILTRATION

Ultrafiltration can be described as a process in which solutes of high molecular weight are retained when the solvent and low molecular weight solutes are forced under hydraulic pressure (between 2 and 10 atmospheres) through a membrane of a very fine pore size, typically between 0.001 and 0.1 µm. It is therefore used for product concentration and purification. A range of membranes made from a variety of polymeric materials, with different molecular weight cut-offs (500–500,000), are available which makes possible the separation of macromolecules such as proteins, enzymes, hormones, and viruses. It is practical only to separate molecules whose molecular weights are a factor of ten different due to variability in pore size. Because the flux through such a membrane is inversely proportional to its thickness, asymmetric membranes are used where the membrane (~0.3 µm thick) is supported by a mesh around 0.3 mm thick. When considering the feasibility of ultrafiltration, it is important to remember that factors other than the molecular weight of the solute affect the passage of molecules through the membranes. There may be concentration polarization caused by accumulation of solute at the membrane surface, which can be reduced by increasing the shear forces at the membrane surface either by conven- tional agitation or by the use of a cross-flow system. Second, slurry of protein may accumulate on the membrane surface forming a gel layer which is not easily removed by agitation. Formation of the gel layer may be partially controlled by careful choice of conditions such as pH. Finally equipment and energy costs may be considerable because of the high pressures necessary; this also limits the life of ultra filtration membranes.

11.9.2 LIQUID MEMBRANES

Liquid membranes are insoluble liquids (eg, an organic solvent) which are selective for a given solute and separate two other liquid phases. Extraction takes place by the transport of solute from one liquid to the other. They are of great interest in the extraction and purification of biologicals for the following reasons:

1. Large area for extraction.
2. Separation and concentration are achieved in one step.
3. Scale-up is relatively easy.

Their use has been reported in the extraction of lactic acid and citric acid using a supported liquid membrane. The utilization of selective carriers to transport specific components across the liquid membrane at relatively high rates has increased interest in recent years. Liquid membranes may also be used in cell and enzyme immobilization, and thus provide the opportunity for combined production and isolation/extraction in a single unit. The potential use of liquid membranes has also been described for the production of alcohol reduced beer as having little effect on flavor or the physicochemical properties of the product.

11.10 DRYING

The drying of any product (including biological products) is often the last stage of a manufacturing process. It involves the final removal of water or other solvents from a product, while ensuring that there is minimum loss in viability, activity, or nutritional value. Drying is undertaken because:
1. The cost of transport can be reduced.
2. The material is easier to handle and package.
3. The material can be stored more conveniently in the dry state.

A detailed review of the theory and practice of drying can be found in Perry and Green (1984). It is important that as much water as possible is removed initially by centrifugation or in a filter press to minimize heating costs in the drying process. Driers can be classified by the method of heat transfer to the product and the degree of agitation of the product. For some products simple tray driers, where the product is placed on trays over which air is passed in a heated oven may be sufficient. A vacuum may be applied to aid evaporation at lower temperatures. In contact driers, the product is contacted with a heated surface. An example of this type is the drum drier (Fig. 11), which may be used for more temperature stable bioproducts. Slurry is run onto a slowly rotating steam heated drum, evaporation takes place and the dry product is removed by a scraper blade in a similar manner as for rotary vacuum filtration. The solid is in contact with the heating surface for 6–15 s and heat transfer coefficients are generally between 1 and 2 kWm^{-}

^{2} K^{-1}. Vacuum drum driers can be used to lower the temperature of drying.

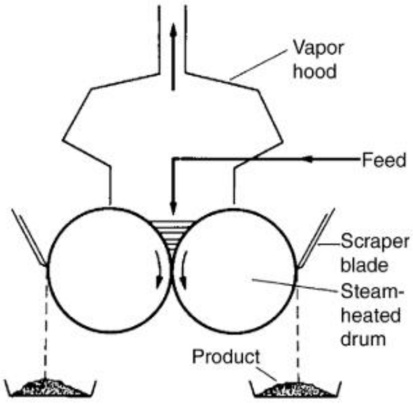


Fig. 11. Cross-Section of a Drum Drier

11.11 CRYSTALLIZATION

Crystallization is an established method used in the initial recovery of organic ac- ids and amino acids, and more widely used for final purification of a diverse range of compounds. Crystallization is a two stage process, the formation of nuclei in a supersaturated solution and crystal growth, which proceed simultaneously and can be independently controlled to some extent. Industrial crystallizers may be batch or continuous processes with super saturation being achieved by cooling or by removal of solvent (evaporative crystallization). In citric acid production, the filtered broth is treated with Ca (OH)2 so that the relatively insoluble calcium citrate crystals will be precipitated from solution. Checks are made to ensure that the Ca (OH)2 has a low magnesium content, since magnesium citrate is more soluble and would remain in solution. The calcium citrate is filtered off and treated with sulfuric acid to precipitate the calcium as the insoluble sulfate and release the citric acid. After clarification with active carbon, the aqueous citric acid is evaporated to the point of crystallization. Crystallization is also used in the recovery of amino acids has reviewed methods for glutamic acid, lysine, and other amino acids. The recovery of cephalosporin C as its sodium or potassium salt by crystallization has been described by Wildfeuer (1985). In 1,3-propanediol fermentations salt by-products (sodium succinate and sodium sul- fate) of the fermentation need to be removed before recovery of 1,3-

propanediol. The recovery is rather than simply removal, of these salts at high yield and purity by batch crystallization. the application of in situ crystallization in by product recover from fermentation broths as soon as the product is formed when such products have an inhibitory or degrading effect on further product formation.

11.12 WHOLE BROTH PROCESSING

The concept of recovering a metabolite directly from an unfiltered fermentation broth is of considerable interest because of its simplicity, the reduction in process stages, and the potential cost savings. It may also be possible to remove the desired fermentation product continuously from a broth during fermentation so that inhibitory effects due to product formation and product degradation can be minimized throughout the production phase. It can also be used to continuously remove undesirable byproducts from a fermentation broth which might otherwise inhibit cell growth or degrade a desired extracellular product. The continuous downstream processing of biopharmaceuticals via centrifugation, filtration, extraction, precipitation, crystallization, and chromatography has been reviewed. A reciprocating plate extraction column (Fig. 11) to use for whole broth processing of a broth containing 1.4 g dm–3 of a slightly soluble organic compound and 4% undissolved solids provided that chloroform or methylene chloride were used for extraction. Methyl-iso-butyl ketone, diethyl ketone, and iso-propyl acetate were shown to be more efficient solvents than chloroform for extracting the active compound, but they presented problems since they also extracted impurities from the mycelia, making it necessary to filter the broth before beginning the solvent extraction. Considerable economies were claimed in a comparison with a process using a Podbielniak extractor, in investment, main- tenance costs, solvent usage, and power costs but there was no significant difference in operating labor costs. An alternative approach is to remove the metabolite continuously from the broth during the fermentation. Cycloheximide production by Streptomyces griseus has been shown to be affected by its own feedback regulation. The tested two techniques at laboratory scale for improving the production of cycloheximide.

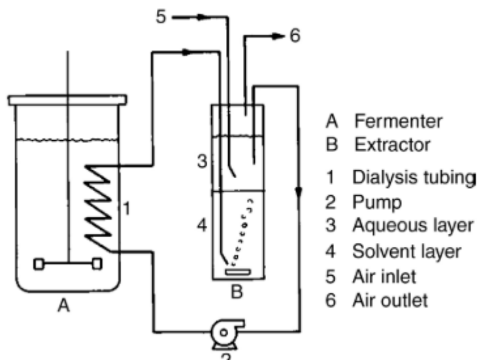


Fig 11. Dialysis-Extraction Fermentation System (Wang et al., 1981)

In a dialysis method, methylene chloride was circulated in a dialysis tubing loop which passed through a 10 dm–3 fermenter. Cycloheximide in the fermentation broth was extracted into the methylene chloride. It was shown that the product yield could be almost doubled by this dialysis-solvent extraction method to over 1200 µg cm–3 as compared with a control yield of approximately 700 pg cm–3. In a resin method, sterile beads of XAD-7, an acrylic resin, as dispersed beads or beads wrapped in an ultrafiltration membrane, were put in fermenters 48 h after inoculation. Some of the cycloheximide formed in the broth is absorbed by the resin. Recovery of the antibiotic from the resin is achieved by solvents or by changing the temperature or pH. When assayed after harvesting, the control (without resin) had a bioactivity of 750 µg cm–3. Readings of total bioactivity (from beads and broth) for the bead treatment and the membrane-wrapped bead treatments were 1420 µg cm–3 and 1790 µg cm–3 respectively. The use of a membrane based system for whole broth processing to separate lysozyme from feed mixture containing lysozyme, myoglobin, and yeast cells for in situ product removal.

Check your Progress -1

Note: Write your answer in the space given below

a. List the physical methods of cell lysis.

b. List out the chromatography technique used for purification of proteins.

…………………………………………………………………………………

11.13 LET US SUM UP

In this unit, you have learnt about the meaning, definition, need, objectives of downstream processing. The methods of purification of industrially valuable products, especially intracellular metabolites have learned. The first step of the process is cell lysis, methods of cell disruption, which includes physical and chemical methods. Further, extraction methods such as chromatography, membrane process, drying and whole broth processing also studied in this chapter. The above basic understanding helps you to become an excellent industrial analytical and quality control personal.

11.14 UNIT - END EXERCISES

1. List out the methods of liquid-liquid extraction.
2. Explain the method of whole broth processing.

11.15 ANSWERS TO CHECK YOUR PROGRESS

1. Homogenization, osmotic lysis, grinding, enzymatic lysis, acid-alkali treatment, ultrasonication are the methods used for the analysis.
2. Adsorption chromatography, ion-exchange, affinity and gel permeases are used for protein purification.

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UNIT -XII: MICROBIAL PRODUCTION OF ORGANIC ACIDS, AMINO ACIDS AND SOLVENTS

Structure

12.1 INTRODUCTION

In this chapter deals with the commercial importance of organic acids (citric acid and Lactic acid), amino acids (L-Glutamic acid and Lysine), and solvents (ethanol and acetone). To understand the microbial metabolism and biochemistry of the desirable end products such as organic acids, amino acids and solvents. This chapter has the elaborate information to know the physical and chemical parameter required for the fermentation of organic acids, amino acids and solvents. The detailed information has been given in brief forms. In addition, the downstream processing of fermented organic acids, amino acids and solvents are of the discussed .

12.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the microbial metabolism to produce the desirable end products such as organic acids, amino acids and solvents.
- Know the physical and chemical parameter required for the fermentation of organic acids, amino acids and solvents.
- Understand the downstream processing of fermented organic acids, amino acids and solvents

12.3 INDUSTRIAL PRODUCTION OF ORGANIC ACIDS

Organic acids are chemical compounds with acidic properties and are widely distributed in nature as normal constituents of all living organisms. Organic acids comprise a key group among the chemical backbone that can be produced by microbial processes. The most common organic acids are the carboxylic acids, (–COOH), and sulfonic acids (–SO_2OH). Organic acids have been used for many years in the chemical, agriculture, food, and pharmaceutical industries. Also, organic acids are used as basic raw material for several industrially valuable solvent productions. A large number of organic acids which includes citric, itaconic, lactic, malic, tartaric, gluconic, mevalonic, salicyclic, gibberelic, diamino-pimelic, and propionic acids are produced through the microbial process with high commercial value. In this chapter the production of citric acid and lactic acid will be discussed.

12.3.1 CITRIC ACID

Citric acid, (2-hydroxy-propane-1,2,3-tricarboxylic acid) derives its name from the Latin word *citrus*, with a molecular weight of 210.14 g/mol, which contains three carboxylic functional groups with three different values of pK_a (3.1, 4.7, and 6.4). Citric acid, a primary metabolic product is the most important organic acid produced in large quantity and is extensively used in food and pharmaceutical industries. Citric acid is a tricarboxylic acid (Figure 1), formed in the tricarboxylic acid cycle (or Krebs cycle) and is found in small quantities in all living organisms. Citric acid was first commercially-produced in England in1826 from lemons (contain 7-9% citric acid) until 1919. It is produced mainly by submerged fermentation using *Aspergillus niger* or *Candida* sp. from different sources of carbohydrates, such as molasses and starch based media.

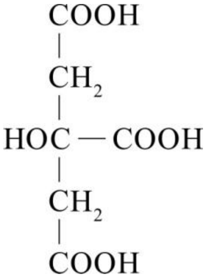


Figure -1: Structure of a Citric acid.

Several studies revealed that, microorganisms such as *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus nidulans*, *Aspergillus fonsecaeus*, *Aspergillus luchensis*, *Aspergillus phoenicus*, *Aspergillus wentii*, *Aspergillus saitoi*, *Aspergillus flavus*, *Absidia* sp., *Acremonium* sp., *Botrytis* sp., *Eupenicillium* sp., *Mucor piriformis*, *Penicillium janthinellum*, *Penicillium restrictum*, *Talaromyces* sp., *Trichoderma viride* and *Ustulina vulgaris* are capable to accumulate citric acid in excess level. Moreover, it is known that several yeasts genera *Candida*, *Hansenula*, *Pichia*, *Debaromyces*, *Torula*, *Torulopsis*, *Kloekera*, *Saccharomyces*, *Zygosaccharomyces* and *Yarrowia* can also produce citric acid from *n*-alkanes and carbohydrates. Based on the economic feasibility, industrial production of citric acid is mainly by submerged fermentation of *Aspergillus* sp. (*A. niger)*. The theoretical yield of citric acid is 112 g (anhydrous citric acid) per 100 g of sucrose. The production has been losses during the trophophase.

USES OF CITRIC ACID

Citric acid has several applications including the food industry, in medicine, pharmacy and in various other industries.

i) **In the food industry:** Citric acid is the major food acidulant used in the manufacture of jellies, jams, sweets, and soft drinks. Also, it is used for artificial flavoring in various foods including soft drinks. Sodium citrate is commonly used in processed cheese manufacture.

ii) **In medicine and pharmacy*:*** Sodium citrate (III) is generally used in blood transfusion to prevent blood clotting. It is a weak acid, is used in efferverscent powers which depend for their efferverscence on the CO_2 produced from the reaction between citric acid and sodium bicarbonate. Also, it is a common primary metabolite presents almost all living organisms, rapidly and completely metabolized to produce energy.

iii) **In cosmetic industry:** Citric acid is used in hair rinses and hair and wig setting fluids. It is used in astringent lotions such as aftershave-lotions because of its low pH.

iv) **Miscellaneous uses*:*** Citric acid is an alternative chemical in the manufacture of detergents in place of phosphates, and reduces the risk of eutrophication (an increase in nutrients which support the growth of aquatic flora). In neutral or low pH conditions the acid has a strong tendency to form complexes hence it is widely used in electroplating, leather tanning, and in the removal of iron clogging the pores of the sand face in old oil wells.

BIOCHEMISTRY OF THE CITRIC ACID PRODUCTION

Citric acid is an intermediate metabolite in the citric acid cycle (TCA). The overproduction or excess accumulation of citric acid requires a unique blend of extraordinary nutritional conditions (example, excess of carbon source, hydrogen ions and dissolved oxygen), which synergistically influence the fermentation process. For example, a deficiency of minerals such as manganese, or phosphate and nitrogen limitation, inhibits the *A. niger* anabolism, and the resulting degradation of proteins which increases ammonium ion (NH_4^+) concentration. High concentrations of NH_4^+ and glucose also repress the synthesis of α-ketoglutarate dehydrogenase, inhibiting the citric acid catabolism via the Krebs cycle, leading to its accumulation.

In another way, an important aspect of the citric acid cycle is the need to completion of the process. When dysfunction represents the citric acid production and accumulation, acetyl-CoA produced from the glycolytic end product of pyruvate by the pyruvate dehydrogenase complex is not only for the citric acid production. Due to the lacking of TCA intermediates, compensation may happened by carboxylation of pyruvic acid by pyruvate carboxylase to form oxaloacetic acid during the idiophase. This reaction requires ATP and is dependent on K^+ and Mg^{2+}, is not the only anaplerotic reaction used to restock the Krebs cycle. Depending on the mutant organism, more oxaloacetic acid can be produced from pyruvic acid by the NADPH-dependent malic enzyme.

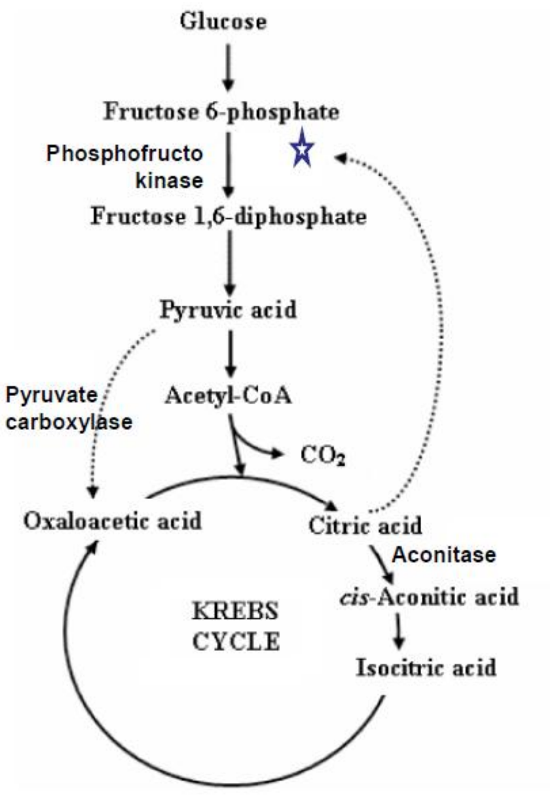


Figure-2: Schematic representation of citric acid production by *A.niger*.

The following are some of such environmental conditions which are applied to increase citric acid production:

1. The concentrations of iron, Mn^{2+}, Mg^{2+}, Zn^{2+}, and phosphate must be limited. To make sure their removal in the medium is treated with ferro-cyanide or by ion exchange fresins. Generally, these metal ions are the prosthetic group of the TCA enzymes: Mn^{2+} or Mg^{2+} are in oxalosuccinic decarboxylase, Fe^{3+} is required for succinic dehydrogenase, while phosphate is required for the conversion of GDP to GTP.
2. Metabolite inhibitors (enzyme inhibitors) also block the metabolic activity and accumulate citric acid. For example, isocitric dehydrogenase is inhibited by ferrocyanide as well as citric acid; aconitase is inhibited by fluorocitrate and succinic dehydrogenase by malonate. The enzyme antagonist may also added to the fermentation to improve the citric acid production
3. The dehydrogenases, especially isocitrate dehydrogenase, are inhibited by anaerobiosis, hence limited aeration is done on the fermentation so as to increase the yield of citric acid.

4. Low pH and especially the presence of citric acid itself inhibits the TCA and hence encourages the production of more citric acid; the pH of the fermentation.

Industrially, mutant strains have been used for citric acid production, excess accumulation in the cell causes leakage of citric acid to its surrounding media. Mutants are lacking certain enzyme of the metabolic pathways; hence the deficiency of the essential enzymes inhibits the flow of the cycle or by the addition of inhibitors or alter the physiological conditions are involve excess citric acid production.

CITRIC ACID PRODUCTION

About 99% of world citric acid production occurs through microbial processes, which can be carried out using submerged or surface cultures. The 70% of anhydrous or monohydrate form of citric acid is used in food and beverage industry as an acidifier or antioxidant to preserve or enhance the flavors and aromas of fruit juices, ice cream, and marmalades. 20% is used in the pharmaceutical industry as antioxidant to preserve vitamins, effervescent, pH corrector, blood preservative, or in the form of iron citrate as a source of iron for the body as well as in tablets, ointments and cosmetic preparations. In the chemical industry, which uses the remaining 10%, is employed as a foaming agent for the softening and treatment of textiles.

For the production of citric acid molasses has been used for a long time by *Aspergillus niger* and occasionally *Asp. wenti.* While several reports suggested that *Penicillium sp., Candida quillermondi*, *Candida oleiphila* , *Candida lipolytica* and *Arthrobacter paraffineus* are used for citric acid production. For submerged fermentation sucrose rich molasses are used, however paraffins are the substrate for surface fermentation.

(a) **Surface fermentation**: using rice bran or other starch or sugar rich substrates have been used for surface fermentation by *Aspergillus niger*, or in liquid solution in flat aluminium or stainless steel pans. *A. niger* can be used to produce citric acid despite the high content of trace metals in rice bran are used. The citric acid is extracted from the bran by leaching and is then precipitated from the resulting solution as calcium citrate.

(b) ***Submerged fermentation***: It is a common practice, where citric acid is made the fermentation the fermentor is made of acid-resistant materials such as stainless steel. The carbohydrate sources are molasses decationized by ion exchange, sucrose or glucose. $MgSO_4$, $7H_2O$ and KH_2PO_4 at about 1% and 0.05-2% respectively are added. The pH is never allowed higher than 3.5. Copper is used at up to 500 ppm as an antagonist of the enzyme aconitase which requires iron. 1-5% of methanol, isopranol or ethanol when added to fermentations containing unpurified materials increase the yield; the yields are reduced in media with purified materials.

As high aeration is deleterious to citric acid production, mechanical agitation is not necessary and air may be bubbled through. Anti-form is added. The fungus occurs as a uniform dispersal of pellets in the medium. The fermentation lasts for five to fourteen days.

EXTRACTION

Traditionally chemical precipitation of citric acid, the process starts with removal of the cell biomass by filtration. Citric acid is precipitated in the form of calcium citrate by the addition of magnesium-free $Ca(OH)_2$. Since magnesium is more soluble than calcium, some acid may be lost in the solution as magnesium citrate if magnesium is added. Calcium citrate is filtered and the filter cake is treated with sulfuric acid to precipitate the calcium in the form of $CaSO_4$. The pH was adjusted between 6.1 and 7.5. The dilute solution containing citric acid is purified by treatment with activated carbon (clarification) and passing through iron exchange chromatography. The purified dilute acid is evaporated to yield crystals of citric acid. Further purification may be required to meet pharmaceutical stipulations.

In counter current extraction of citric acids, various solvents are used to solublize or extract the citric acid from the aqueous culture filtrate. More than 70 to 80% of citric acid is extracted through this method (Fig.2). After the clarification of fermented broth the supernatant/ filtrate are subjected to solvent extraction. Different solvents have been used such as hexane, acetone, ether solvents etc. After the extraction the concentrated citric acid is reextracted by amine extraction, the rafinate is again extracted by the same method to increases the extraction efficacy.

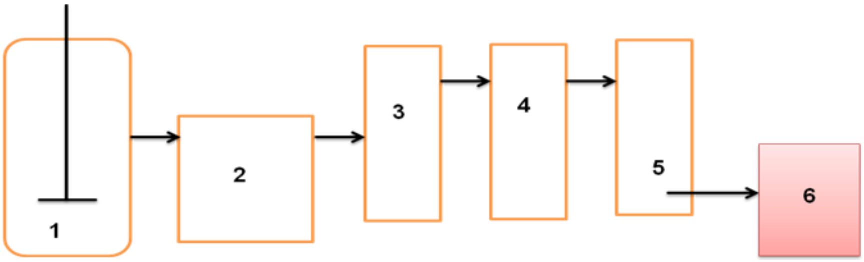


Figure -2 **Counter current extraction of citric acids.** 1. Fermenter; 2. Clarification tank (remove the solids); 3. Solvent extraction & precipitation of citric acid [concentrating citric acid to >80% of the saturation value]; 4) aqueous extraction; 5) precipitation, 6) Drying & packaging.

Compressed CO_2 was then dissolved in this mixture to remove the residual impurities as precipitates using the antisolvent effect of CO_2. The deposited impurities were readily separated from the acetone solution of citric acid by a settler. Finally, food additive grade citric acid was easily obtained by simple decolorization and crystallization methods.

12.3.2 LACTIC ACID

Lactic acid fermentation is a anaerobic metabolic process by which glucose and other six-carbon sugars are converted into lactate or lactic acid and cellular energy. Lactic acid fermentation process usually happened in facultative anaerobic organisms. Sometimes when oxygen is present aerobic metabolism is happening, if pyruvate is building up faster than it can be metabolized, the fermentation will happen anyway. Lactic acid fermentation is an enzymatic process by Lactate dehydrogenase, which catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. There are two different class of lactic acid fermentation carried out by microorganisms, they are (i) homolactic fermentation (single end product- lactic acid), and (ii) heterolactic fermentation, in contrast, yields carbon dioxide and ethanol in addition to lactic acid, in a process called the phosphoketolase pathway. Lactic acid is produced by many organisms: animals including man produce the acid in muscle during work.

CHEMICAL PROPERTIES OF LACTIC ACID

Lactic acid is a three carbon organic acid: one terminal carbon atom is part of an acid or carboxyl group; the other terminal carbon atom is part of a methyl or hydrocarbon group; and a central carbon atom having an alcohol carbon group.

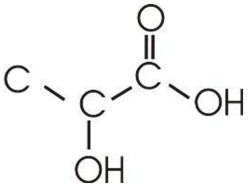


Figure – 3: Structure of Lactic acid, $C_3H_6O_3$.

Lactic acid exists in two optically active isomeric forms (L and D forms), and it is soluble in water and water miscible organic solvents but insoluble in other organic solvents. Lactic acid display low volatility. It reacts with alcohol or esters or amides. The reaction undergoes xanthation with carbon bisulphide, esterification with organic acids and oxygenation or dehdrogenation to form derivatives of pyruvic acid. The physical properties of the lactic acid is given in table-1.

APPLICATIONS OF LACTIC ACID

Industrial grade lactic acid is used as an acidulant in vegetable and leather tanning industries. Other small scale applications of lactic acid such as pH adjustment, hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyl resin modifier, solder flux, textile printing, electroplating and electropolishing baths, and detergent making.

Table - 1 Physical Properties of Lactic acid.

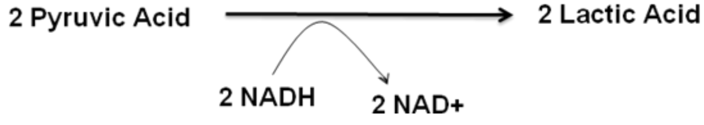
Properties	Iso-forms	Range
Melting point (^oC)	L or D form	52.7 – 53
	Racemic	16.4 – 18.0
Boiling point (^oC)	L or D form	103
	Racemic	122
Solid density (g ml^{-1})	--	1.33
Liquid density (g ml^{-1})	88% (wt)	1.201
pKa	--	3.79 -3.86
	Racemic	3.73
Soluble in	Water, alcohol, furfurol	
Insoluble in	Chloroform, petroleum ether, and carbon disulfide	

In addition, lactic acid has numerous pharmaceutical and cosmetic applications such as formulations of topical ointments, humectants, lotions, anti acne solutions, parenteral solutions, anti carries agents, and dialysis applications. For example, calcium lactate is used for calcium deficiency therapy. Other than that biodegradable polymer of lactic acid have medical applications such as orthopedic implants, controlled drug release, etc. Esters of lactic acids like ethyl/butyl lactate is used as green solvents due to its high boiling, non-toxic and degradable properties. Moreover, low degree polymers of poly L-lactic acid are used in large-scale agricultural applications (degradable mulch films).

LACTIC ACID METABOLISM

Lactic acid was the first organic acid produced by commercial fermentation. Most of the organisms produce either the D-or the L-form of the acid. However, *Lactobacillus plantarum* produce both D- and L-form of lactic acid. When both the D- and L- form of lactic acid are mixed it to a racemic mixture (DL), which is optically inactive. Metabolically, lactic acid is derived from enzymatic conversion of pyruvate, which is reduced to lactic acid. This is a single step reaction carried out by Lactic acid bacteria (LAB). There are two types of lactic acid fermentation. Organism ferment lactic acid by either i) Homo lactic fermentation or ii) Hetero lactic fermentation (fig-4).

Homolactic fermentation: In homolactic fermentation, end product is the lactic acid. Pyruvate is reduced to lactate or lactic acid by the enzyme lactate dehydrogenase (Pyruvate reductase). the bacteria such as *Streptococcus thermophiles, Streptococcus lactis, lactobacillus lactis, Lactobacillus bulgarius, Pediococcus,* and *Enterococcus sp.* ferment lactic acid as the single end product. Lactic acid fermentation converts the 3-carbon pyruvate to the 3-carbon lactic acid ($C_3H_6O_3$) and regenerates NAD^+ in the process, allowing glycolysis to continue to make ATP in low-oxygen conditions. Lactic acid is formed by the reduction of pyruvate.



Homofermentative bacteria convert glucose to two molecules of lactate and use this reaction to perform substrate-level phosphorylation to make two molecules of ATP:

Glucose + 2 ADP + 2 P_i → 2 lactate + 2 ATP

Homolactic fermentation is important in dairy industry for souring of milk to produce various fermented products. *Streptococcus mutant*, bacteria responsible for dental caries is a homolactic fermenting bacteria. *Lactobacillus* spp (probiotic) in the digestive tract of human helps in digestion of lactose present in milk.

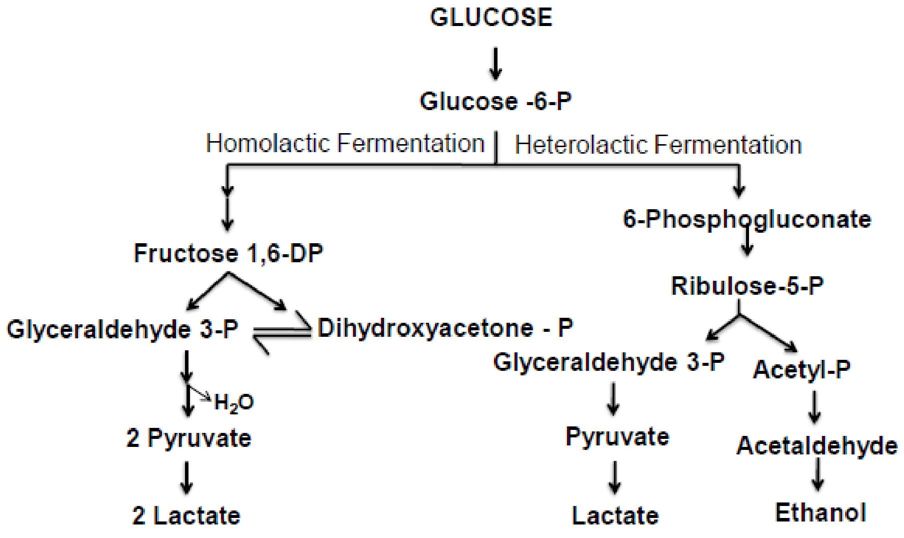


Figure-4 Pathway of homolactic and heterolactic fermentation.

Heterolactic fermentation: In hetero lactic fermentation, lactic acid, ethanol and CO_2 are end product. Lactic acid and the ethanol are produced via the 6-P-gluconate intermediate (Pentose phosphate pathway). The 3-carbon glyceroldehyde-3-P is converted to pyruvate and further reduced to Lactic acid. The 2-carbon acetyl phosphate is further converted to acetaldehyde and ethanol. The bacteria such as *Leuconostoc mesenteroides, Lactobacillus bifermentous,* and *Leconostoc lactis* are produce lactic acid and ethanol via the heterolactic fermentation.

Heterofermentative bacteria produce less lactate and less ATP, but produce several other end products:

Glucose + ADP + P_i → lactate + ethanol + CO_2 + ATP

INDUSTRIAL FERMENTATION OF LACTIC ACID

There are many microorganisms can produce lactic acid, the amount of lactic acid production is too small, hence the efficient and higher producing homolactic fermenting lactic acid bacteria, *Lactobacillus* spp., especially *L. delbruckii* is used. In addition to this industrial bacteria, a fungi organisms which produce adequate amounts and are therefore used in industry are the *Rhizopus oryzae* is also being in use. Both organisms produces the L-form of the lactic acid, compared with Lactobacillus, *Rhizopus* has the merits of being much shorter in duration of fermentation, and easy purification of lactic acid.

Acids are corrosive in nature. Hence the fermenter of lactic acid fermentation is usually between 25,000 and 110,000 liters in capacity is made of wood. Alternatively special stainless steel (type 316) may be used. The fermenter and the media are sterilized by steaming before inoculation. Usually the thermophilic clostridia can contaminate the media yielding butanol and butyric acid.

In lactic acid fermentation by Lactobacillus delbrueckii NRRL B-445, the production media contains 90 g l^{-1} glucose and 20 g Pl^{-1} CSL as a sole nutrient source (table-2). Maximum yield of around 70.7 g l^{-1} lactic acid was obtained with an economic efficiency of 98 g lactic acid per/100g of nutrient. The fermentation media is inoculated with the homolactic *Lactobacillus delbrueckii* NRRL B-445 at 5% inoculums, calcium carbonate is added to the medium to maintain the pH at around 5.5-6.5.

Table- 2 CSL media components [Mercier *et al.*1992]

Ingredient	**g/L**
Yeast extract	5
peptone	10
sodium acetate	5
sodium citrate	2
K_2HPO_4	2
$MgSO_4.7H_2O$	0.58
$MnSO_4.H_2O$	0.12
$FeSO_4.7H_2O$	0.05
pH	5.5-6.5

The carbon source used in the fermentation media has varied widely. They included whey, sugars in potato and corn hydrolysates, sulfite liquour, and molasses. Due to the recovery issues, high quality

lactic acid preparation, purified sugar and a minimum of other nutrients are used. *Lactobacillus sp.* requires additional vitamins and growth factors for growth. To assist recovery of lactic acid the initial sugar content of the broth is not higher than 12% to enable its complete utilization at the end of 72 hours. Fermentation with *Lactobacillus delbruckii* is usually for 5 to 10 days whereas with *Rhizopus oryzae*, it is about two days.

Generally, lactic fermentation is anaerobic, the organisms used are facultative anaerobes, complete anaerobiosis is not necessary for the fermentation. The incubation temperature is slightly higher than the normal bacterial growth, and it is around 45°C. Therefore contamination is not a problem, except by thermophilic clostridia.

LACTIC ACID EXTRACTION & PURIFICATION

The main problem in lactic acid production is the recovery of the acid. Lactic acid is crystallized with great difficulty and in low yield. The purest forms are usually colorless syrups which readily absorb water. At the end of the fermentation when the sugar content is about 0.1%, the fermented media is pumped into settling tanks, which contains calcium hydroxide at pH 10, during the mixing process clear calcium lactate is allowed to settle. Then it is filtrated from the slurry. It is then treated with sodium sulfide, decolorized by adsorption with activated charcoal, acidified to pH 6.2 with lactic acid and filtered. The calcium lactate liquor may then be spray-dried. Purification protein is showed in the fig-5.

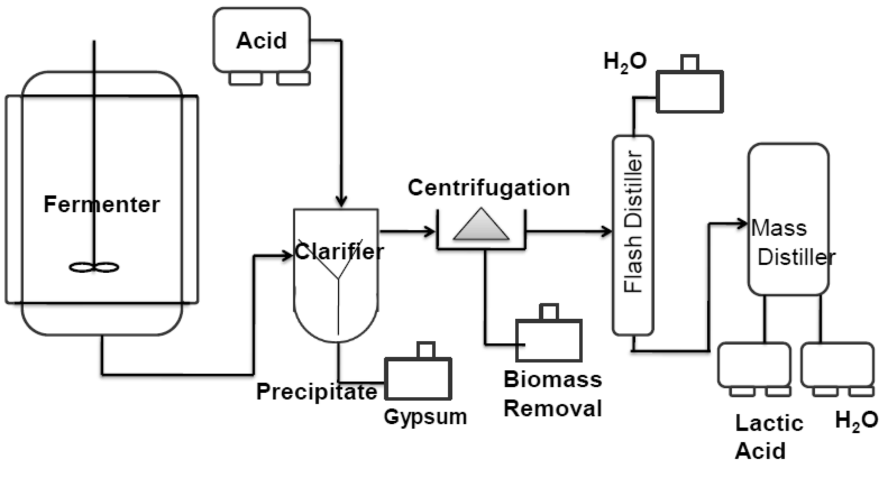


Fig-5 Purification of Lactic acid.

For technical grade lactic acid (44-45% total acidity) the calcium is precipitated as $CaSO_4.2H_2O$ which is filtered off. However in food grade acid has a total acidity of about 50%. It is made from the fermentation of higher grade sugar and bleached with activated carbon. Metals especially iron and copper are removed by treatment with ferrocyanide. It is then filtered. In plastic grade preparation lactic acid is esterified with methanol after concentration. High-grade lactic acid is made by various methods: steam distillation under high vacuum, solvent extraction etc.

Check your Progress-1
Note: Write your answer in the space given below
a. Name the commercial producer of citric acid.
b. Discuss the uses of lactic acid.
....

12.4 MICROBIAL PRODUCTION OF AMINOA ACIDS

Amino acids are produced at the multi-million-ton-scale with fermentative production of l-glutamate and l-lysine alone being estimated to amount to more than five million tons in the year 2013. Metabolic engineering constantly improves productivities of amino acid producing strains, mainly *Corynebacterium glutamicum* and *Escherichia coli* strains. Classical mutagenesis and screening have been accelerated by combination with intracellular metabolite sensing. Synthetic biology approaches have allowed access to new carbon sources to realize a flexible feedstock concept. Moreover, new pathways for amino acid production as well as fermentative production of non-native compounds derived from amino acids or their metabolic precursors were developed. These include dipeptides, α,ω-diamines, α,ω-diacids, keto acids, acetylated amino acids and ω-amino acids. In this chapter, microbial production of L-Glutamic acid and L-lysine are discussed below.

12.4.1 L-GLUTAMIC ACID

L-amino acids are major biological components commercially used as additives in food, feed supplements, infusion compounds, thera-peutic agents and precursors for peptides synthesis or agriculture based chemicals. The amino acids are the second most important category, after antibiotics, with fermentation products exhibiting the

highest growth rates. L-glutamic acid was the first amino acid produced commercially. The substance was discovered and identified in the year 1866 by the German chemist Karl Heinrich Leopold Ritt-hausen. L-glutamic acid was mainly produced by microbial fermentations and the chemical mode of synthesis is not widely preferred due to the formation of racemic mixture.

It has a formula $C_5H_9O_4N$ (FIG.1). Its molecular structure could be idealized as $HOOC-CH(NH_2)-(CH_2)_2-COOH$, with two carboxyl groups -COOH and one amino group $-NH_2$ (Fig-6). However, in the solid state and mildly acid water solutions, the molecule assumes an electrically neutral zwitterion structure $^{-}OOC-CH(NH^{+}_{3})-(CH_2)_2-COOH$. It is encoded by the codons GAA or GAG.

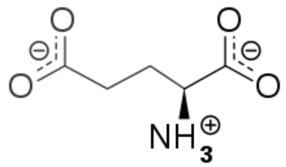


Figure-6: Structural formula of L-glutamic acid.

Glutamic acid is produced on the largest scale of any amino acid, with an estimated annual production of about 1.5 million tons in 2006. Chemical synthesis was supplanted by the aerobic fermentation of sugars and ammonia in the 1950s, with the organism *Corynebacterium glutamicum* (also known as *Brevibacterium flavum*) being the most widely used for production. Isolation and purification can be achieved by concentration and crystallization; it is also widely available as its hydrochloride salt. L-glutamic acid is produced per year using coryneform bacteria. In biotechnological processes, *Corynebacterium* species are used for economic production of glutamic acid by submerged fermentation. A number of fermentation techniques have been used for the production of glutamic acid. Glucose is one of the major carbon sources for production of glutamic acid. However, glutamic acid was produced with various kinds of raw materials using sub-merged fermentation of palm waste hydrolysate, cassava starch, sugar cane bagasse, date waste.

USES OF L-GLUTAMIC ACID

- L-glutamic acid is an excitatory neurotransmitter increasing the firing of neurons in the human central nervous system. Moreover, Glutamic acid is recognized as a major excitatory neurotransmitter in the human brain and in the spinal cord, transformted into

Glutamine or Gamma-Aminobutyric Acid. This amino acid is necessary for proper cell functioning, but is considered as a non-essential amino acid, because human body is able to produce it.

- In food industries L-Glutamic Acid is widely used as nutritional supplement in food production.
- In beverages - L-Glutamic Acid is widely used as flavor enhancer in beverage
- Flavor enhancer in MSG and spices to improve flavor.
- As nutritional supplement in food industries, animal feed.
- Act as a feed stock chemical for various industrial fermentations.
- In cosmetics: it widely used as hair restore, in treatment in hair loss. It used as anti wrinkle substance, used as anti aging substance.

BIOSYNTHESIS OF L-GLUTAMIC ACID

The pathway for the synthesis of glutamic acid with glucose as the carbon source is depicted in Fig.7. Glucose is broken down to phosphoenol pyruvate and then to pyruvate. Pyruvate is converted to acetyl CoA. Phosphoenol pyruvate (by the enzyme phosphoenol pyruvate carboxylase) can be independently converted to oxaloacetate. Both these carboxylation reactions are quite critical, and require biotin as the cofactor.

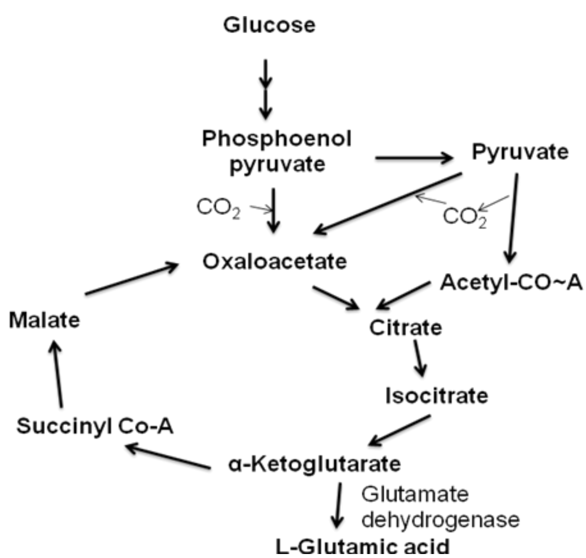


Figure-7: Metabolic path way of L-glutamic acid production.

The next series of reactions that follow are the familiar citric acid (Krebs) cycle reactions wherein the key metabolite namely α-ketoglutarate is produced. In the routine citric acid cycle, α –

ketoglutarate is acted upon by the enzyme α- ketoglutarate dehydrogenase to form succinyl CoA.

For the production of glutamic acid, α-ketoglutarate is converted to L-glutamic acid by the enzyme glutamate dehydrogenase (GDH). This enzyme is a multimer, each subunit with a molecular weight of 49,000. The reducing equivalents, in the form of NADPH + H^+, are required by GDH. They are generated in the preceding reaction of Krebs cycle (catalysed by the enzyme isocitrate dehydrogenase) while converting isocitrate to α-ketoglutarate. The supply and utilization of NADPH + H^+ occurs in a cyclic fashion through the participation of the two enzymes, namely isocitrate dehydrogenase and glutamate dehydrogenase (Fig.8).

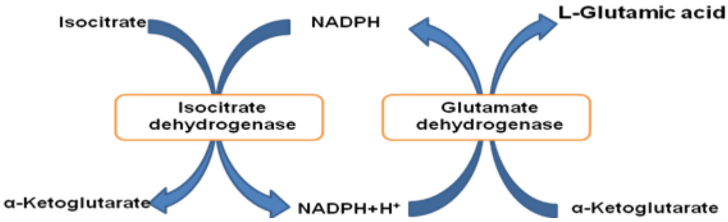


Figure-8: Enzymatic conversion of α-ketoglutarate.

Theoretically, one molecule of glutamic acid can be formed from one molecule of glucose. In practice, the conversion efficiency of glucose to glutamic acid was found to be around 70%.

REGULATION OF GLUTAMIC ACID BIOSYNTHESIS

The essential requirement for glutamic acid production is the high capability for the supply of the citric acid cycle metabolites. This is made possible by an efficient conversion of phosphoenol pyruvate as well as pyruvate to oxaloacetate .Thus, there are two enzymes (phosphoenol pyruvate carboxylase and pyruvate carboxylase) to efficiently produce oxaloacetate, while there is only one enzyme (pyruvate dehydrogenase) for the formation of acetyl CoA.

Certain microorganisms which have either phosphoenol pyruvate carboxylase (e.g., E. coli) or pyruvate carboxylase (e.g. *B. subtilis*) are not capable of producing glutamic acid to any significant extent. C. glutamicum has both the enzymes and therefore can replenish citric acid cycle intermediates (through oxaloacetate) while the synthesis of glutamic acid occurs. Another key enzyme that can facilitate optimal production of glutamic acid is α-ketoglutarate dehydrogenase of citric acid cycle. Its activity has to be substantially low for good synthesis of glutamic acid, as is the case in C.

glutamicum. Further, exposing the cells to antibiotics (penicillin) and surfactants reduces the activity of α-ketoglutarate dehydrogenase while glutamate dehydrogenase activity remains unaltered. By this way, oxidation of α-ketoglutarate via citric acid cycle can be minimised, while the formation of glutamic acid is made maximum possible.

Release of Glutamic Acid: Glutamic acid is synthesized intracellularly, and therefore its release or export is equally important. It now appears that there is a carrier-mediated energy-dependent active process involved for the export of glutamic acid. There are several ways of increasing the membrane permeability for exporting glutamic acid***:***

1. Biotin limitation
2. Addition of saturated fatty acids
3. Addition of penicillin
4. Use of oleic acid auxotroph's
5. Use of glycerol auxotroph's
6. Addition of local anesthetics
7. Addition of surfactants (Tween 40).

The effect of biotin deficiency in facilitating the release of intracellular glutamic acid has been worked out. Biotin is an essential cofactor (required by the enzyme acetyl CoA carboxylase) for the biosynthesis of fatty acids. Due to a limited supply or deficiency of biotin, fatty acid biosynthesis and consequently phospholipid synthesis is drastically reduced. As a result, membrane formation (protein- phospholipid complex) is defective which alters permeability for an increased export of intracellular glutamic acid.

MICROBIAL PRODUCTION OF L-GLUTAMIC ACID

(i) *Organisms*: Wild type strains of the organisms of the four genera mentioned above are now used for the production of glutamic acid. The preferred organism is however *Corynebacterium glutamicum.* The properties common to the glutamic acid bacteria are:

(a) they are all Gram-positive and non-motile;

(b) they require biotin to grow;

c) they lack or have very low amounts of the enzyme α-ketoglutarate, which is formed by removal of CO_2 from isocitrate formed in TCA cycle (citric acid cylce). Since α -ketoglutarate is not dehydrogenated it is available to form glutarate by reacting with ammonia.

Carbon source requirements*:* Carbon sources such as glucose, fructose, sucrose, maltose, ribose or xylose can be utilized by the GA producing bacteria as the substrate for cell growth and GA biosynthesis. A crude carbon source (feedstock) has historically been avoided because high levels of extraneous materials can cause troublesome separation problems in the recovery stage. In the late 1950, dextrose from corn starch was the most commonly used feed stock. Other feed stocks like concentrated whey, hydrolysed potato, cellulosic material, sulphite liquor, and molasses were also used. Fermentation route from renewable resources like sugarcane juice with suitable microorganisms has always been favoured to produce optically pure L-(+) glutamic acid as a myriad of value-added products derived from biological origin are readily accepted by food industries and consumers. In addition to that sugarcane juice is easily available throughout the year in some major sugar cane growing countries like India and Brazil.

Other nutrients: Addition of nutrient supplementation like yeast extract, peptones and other micro-macro inorganic nutrients required for the growth of micro-organism in fermentation broth, leads to increased sugar utilization and reduced fermentation time but it also adds to residual impurities in fermentation broth. To produce pure and monomer grade GA efficient separation of those impurities is essential. Ammonium ion and urea are detrimental to both cell growth and product formation and its concentration in the medium must be maintained at a low level. To neutralize the acid that is formed during fermentation, calcium carbonate, calcium hydroxide and gaseous ammonia are typically used.

Nitrogen Source: Ammonium sulphate, ammonium chloride, ammonium phosphate, aqueous ammonia, ammonia gas and urea have been used as nitrogen source. Although large amount of ammonium ions are necessary, a high concentration of it inhibits the growth of the microorganism as well as the yield of L-glutamic acid. Therefore, suitable amount of ammonia is added, as the fermentation progresses. These salts also help in the pH control.

Growth Factors: The important growth factor is biotin. Its optimal concentration depends upon the carbon source used. In media with 10% glucose, its requirement is 5 mg liter^{-1}. In media with lower glucose concentration, it is considerably lower. Some strains require L-cystine as an additional growth factor.

Oxygen Supply: The oxygen concentration should neither be too low nor too high. Optimal L-glutamic acid yields are obtained at kb value of 3.5 x 10^{-6} mole 02 atm^{-1} min^{-1} ml^{-1}. Excretion of lactate and succinate occurs under oxygen deficiency, whereas excess oxygen under ammonium ions deficiency causes growth inhibition and production of α-ketoglutarate. In both the cases, glutamic acid yields are low.

pH & Temperature: Optimum pH for growth and glutamic acid production is 7.0-8.0 and it is controlled by the addition of ammonium salts. The optimal temperature is 30° to 35° and a high degree of aeration is necessary.

Table-3 Media composition

Components	In % (g)
Glucose,	10
Corn steep liquor	0.25
Enzymatic casein hydrolysate	0.25
K_2HPO_4	0.1
Mg. SO_4, $7H_2$ O	0.25
Urea,	0.5.
Biotin	2.5 mg/lit

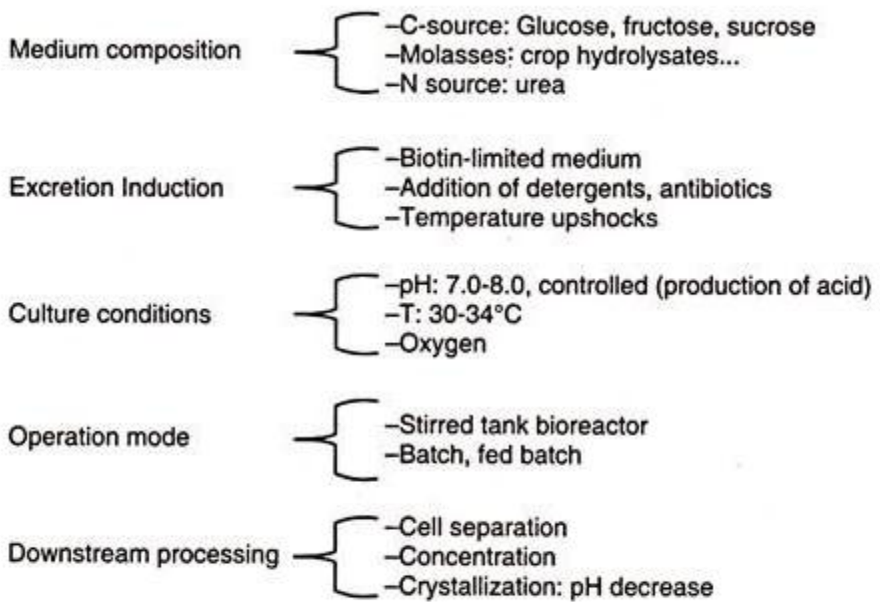


Figure 9: Important components for L-Gutamic acid production.

HARVEST AND RECOVERY

The same process of recovery that is employed for L-lysine is also employed for the harvest and recovery of L-glutamic acid. Glutamic

acid can also be produced through biotransformation of racemic mixture of D-and L-hydantoin-5-propionic acid with the help of hydantoinase. Simultaneously D-hydantoin is converted to L-hydantoin-5-propionic acid in the presence of hydantoin racemase. The flow diagram of the commercial production of L-glutamic acid is described in Fig.10.

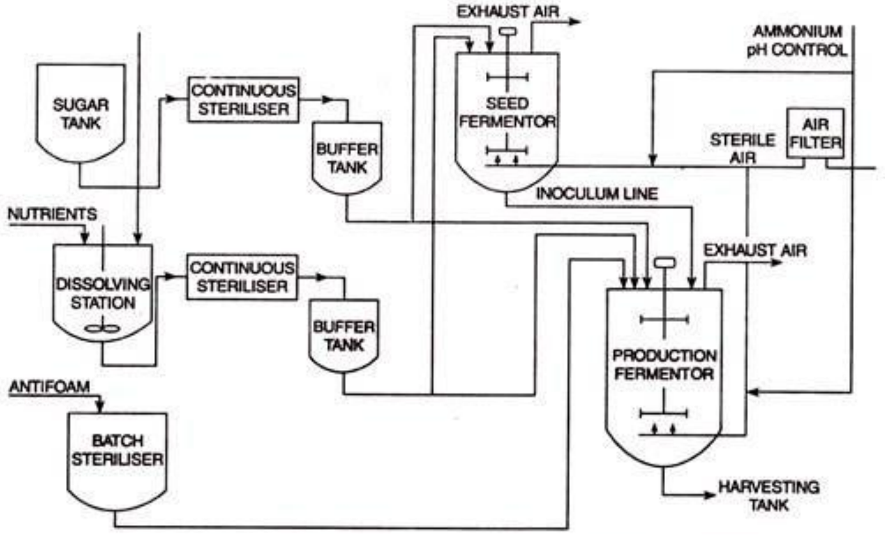


Figure 10: A flow diagram for the commercial production of L-glutamic acid.

12.4.2 L-Lysine

Lysine is an essential, economically important amino acid used as food and feed supplement. It has also some pharmaceutical applications in the formulation of diets with balanced amino acid composition and in amino acid infusions. Chemical, enzymatic and fermentation processes have been used to synthesize lysine.

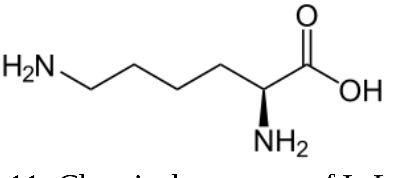


Figure 11: Chemical structure of L-Lysine.

Generally, natural strains cannot produce industrially significant amounts of L-lysine in the fermentation medium due to various metabolic regulation mechanisms. However, alteration of these mechanisms can lead to L-lysine production and accumulation. Two

distinct biosynthetic pathways are known for L-lysine production. In certain actinomycetes fungi and algae the carbon skeleton of L-lysine arises from acetate and α-ketoglutarate by biosynthetic sequences that include α-amino adipic acid. The other pathway has been found in bacteria, higher plants, blue green algae and certain fungi (some phycomycetes) and protozoa. The L-lysine carbon chain is synthesized from pyruvate and aspartate and α-ε-diaminopimelic acid is a key intermediate. The metabolic pathway is illustrated in Fig. 12.

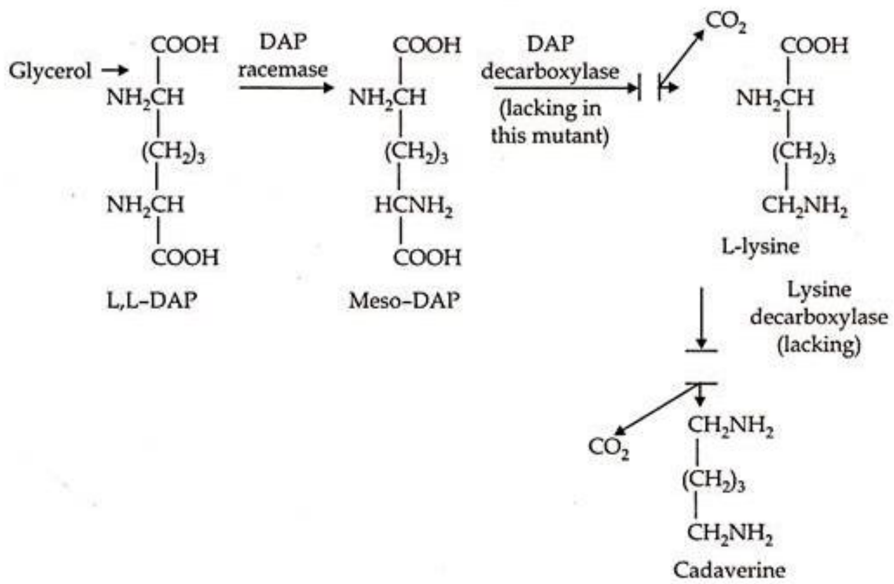


Figure 12: Metabolic path way of an *E.coli* auxotroph which produce high amount of diaminopimelic acid (DAPA) during growth on glycerol.

In the DAP pathway is found in both prokaryotes and plants and begins with the dihydrodipicolinate synthase (DHDPS) catalysed condensation reaction between the aspartate derived, *L*-aspartate semialdehyde, and pyruvate to form (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid (HTPA). The product is then reduced by dihydrodipicolinate reductase (DHDPR), with NAD(P)H as a proton donor, to yield 2,3,4,5-tetrahydrodipicolinate (THDP). From this point on, there are four pathway variations found in different species, namely; the acetylase, aminotransferase, dehydrogenase, and succinylase pathways. These four variant pathways converge at the formation of the penultimate product, *meso*-diaminopimelate, which is subsequently enzymatically decarboxylated in an irreversible reaction

catalysed by diaminopimelate decarboxylase (DAPDC) to produce *L*-lysine.

The DAP pathway is regulated at multiple levels, including upstream at the enzymes involved in aspartate processing as well as at the initial DHDPS catalysed condensation step. Lysine imparts a strong negative feedback loop on these enzymes and, subsequently, regulates the entire pathway.

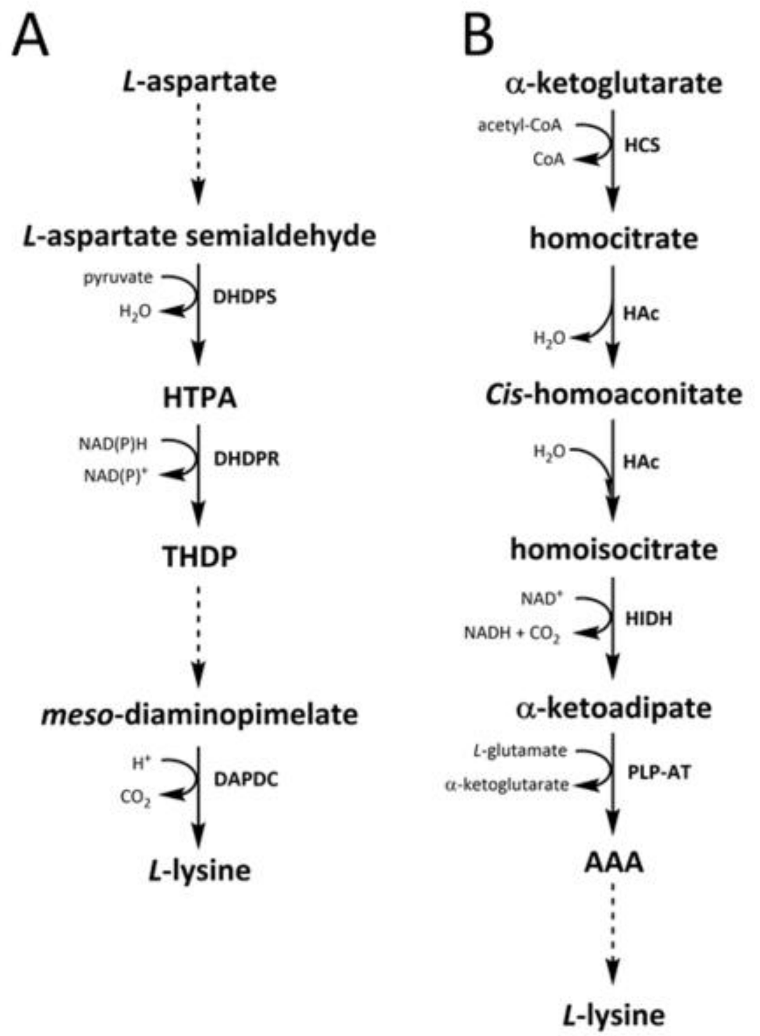


Figure 12 a) DAP Pathway b) AAA pathway

The AAA pathway is common in several yeast species and certain bacteria (*Thermus thermophilus* and *Pyrococcus horikoshii)*, it involves the condensation of α-ketoglutarate and acetyl-CoA via the intermediate AAA for the synthesis of *L*-lysine. The rate-limiting step in the AAA pathway is the condensation reaction between acetyl-CoA

and α-ketoglutarate catalysed by homocitrate-synthase (HCS) to give the intermediate homocitryl-CoA, which is hydrolysed by the same enzyme to produce homocitrate. Homocitrate is enzymatically dehydrated by homoaconitase (HAc) to yield *cis*-homoaconitate. HAc then catalyses a second reaction in which *cis*-homoaconitate undergoes rehydration to produce homoisocitrate. The resulting product undergoes an oxidative decarboxylation by homoisocitrate dehydrogenase (HIDH) to yield α-ketoadipate. AAA is then formed via a pyridoxal 5′-phosphate (PLP)-dependent aminotransferase (PLP-AT), using glutamate as the amino donor.

1. In fungi, the AAA is reduced to α-aminoadipate-semialdehyde via AAA reductase in a unique process involving both adenylation and reduction that is activated by a phosphopantetheinyl transferase, to form semialdehyde. It can be reduced with glutamate to form saccharopine, finally dehydrogenation and oxidative deamination resulting in *L*-lysine.
2. In prokaryotes, AAA is first converted to *N*-acetyl-α-aminoadipate, which is phosphorylated and then reductively dephosphorylated to the ε-aldehyde. The aldehyde is then transaminated to *N*-acetyl-lysine, which is deacetylated to give *L*-lysine.

APPLICATIONS OF L-LYSINE

L-lysine is an important aminoacid for making the proteins, cellular architecture, and it is useful in many fields:

1. L-lysine is an essential amino acid required for the human nutrition.
2. It is used as supplementary for cereal proteins.
3. Protein quality of certain foods like wheat (based foods) is improved by addition of L-lysine which results in the improved growth and tissue synthesis.
4. It is used as a nutraceutical.
5. It is used as animal feed supplements mainly poultry, aquaculture and cattle’s.

FERMENTATION OF L-LYSINE

Fermentation Process of L-Lysine consists of four stages. They are:

1. Preparation of inoculum,
2. Preparation of medium,
3. Fermentation process,
4. Harvest and recovery,

(i) Preparation of Inoculum: Suitable and high yielding mutant strain of *C. glutamicum* is used from the stock culture for the production of inoculum. Different microorganisms have been used for the industrial production of L-lysine, which includes *Brevibacterium flavum, Corynebcterium glutamicum, Brevibcterium lactofermentum* and *Nocardia* spp. Seed cultures of the production strains grow in glucose or acetate media.

Table 4 (a) The medium for first seed culture contains:

Glucose	**2%**
Peptone	**1%**
Meal extract	**0.5%**
NaCl	**0.25%**

The medium is prepared in tap water.

(b) The medium for second seed culture contains:

Cane molasses	**5%**
$(NH4)_2SO_4$	**2%**
Corn steep liquor	**5%**
$CaCO_3$	**1%**

(ii) Preparation of Medium: The medium with the following composition is used as fermentation medium. Reducing sugar (expressed as inverted cane molasses), 20%, Soyabean meal hydrolysate (as weight of meal before hydrolysis with $6NH_2SO_4$ 1.8% and neutralization with ammonia water) are dissolved in tap water and sterilized. cane molasses is used for C. glutamicum, Acetic acid is used for Brevibacterium spp. and n-paraffin is used for Nocardia based L-lysine production.

(iii) Fermentation Process: The fermentation is carried out at 28°C and is allowed upto 60 hours. The amount of growth factors, homoserine or threonine and methionine should be appropriate for the production of L-lysine and suboptimal quantity to support the optimal

growth. The biotin concentration in the medium should be greater than 30 mg per liter. If biotin is supplied in limited quantities there will be accumulation of L- glutamic acid instead of L-lysine. Cane molasses generally supplies enough biotin. There will be 30-40% yield of L-lysine as monohydrochloride in relation to the initial sugar concentration. Foam production in the aerated culture can be controlled by adding suitable antifoam agent.

Product Recovery: The same process of recovery of L-lysine that is employed in indirect fermentation process is also used in this process. Mutant strains of Bacillus licheniformis are also employed for the production of L-lysine. The mutant strains were obtained by the introduction of both analogue-resistance and auxotrophy. The medium containing 10% cane molasses is used. A temperature of 40°C is suitable for L- lysine production. The sporulation activity which reduces yield, can be suppressed by the addition of certain antibiotics like tetracycline and chloramphenicol. These mutants yield approximately 30 mg of L-lysine per ml of carbon source used.

After sufficient quantities of L-lysine is formed, lysed bacterial cells are removed from the fermentation broth by centrifugation. The L-lysine is obtained in pure form after acidification by any one of the following separating processes:

1. Precipitation at the isoelectric point
2. Ion exchange chromatography
3. Electrophoresis
4. Extraction with organic solvents

Enzyme mediated production of L-lysine.

L-lysine is also produced by enzyme process. Racemase mixture of D and L-amino- caprolactum can be transformed by the L-α-aminocaprolactum hydrolase to lysine. Racemase enzyme converts D-α-aminocaprolactum to L-α-aminocaprolactum (Fig. 13). The L-α amino-caprolactic hydrolase and racemase enzymes are obtained from the bacteria, Achromobacter obae and yeast, Cryptococcus lauranti.

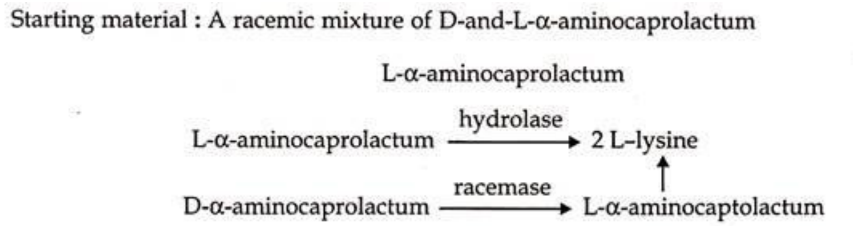


Figure 13: Production of L-lysine through biotransformation of Achromobacter spp.

12.5 SOLVENT PRODUCTION

The flexibility of bacteria in the production of commercially useful products specifically organic solvents is well represented by the fermentations that produce butanol, acetone, isopropanol, 1,3- and 1,2-propanediol, and 2,3-butanediol. Most of these solvents can be synthesized from petroleum-derived feedstock chemicals. The advantage of industrial applications of these fermentations ultimately depends on the economics of the bioprocesses or the need for a chiral product, which is more easily achieved through a bioprocess. Butanol, acetone, and isopropanol were traditionally used as solvents, and the industrial fermentation producing these chemicals was thus known as the solvent fermentation. Solvent fermentation is performed by several species of Clostridia, and it was the first industrial fermentation utilizing pure cultures and aseptic techniques. Its large scale was also unprecedented. Butanol has desirable properties as an automobile fuel, and this potential use has received much attention. Current efforts in improving butanol fermentation for industrial uses focus on the development of less expensive raw materials, a higher final product concentration, and bacterial strains that are more amenable to genetic manipulations. In this chapter, microbial production of ethanol and acetone are discussed.

12.5.1 ETHYL ALCOHOL

The ethyl alcohol from agricultural raw materials to decrease the utilization of crude oil has recently received great impact on national and international levels. In the mid-1900s, microbial production of ethanol was replaces synthetic ethanol from petroleum. The microbiologists started the process of microbial production of ethanol as industrial feedstocks or solvents such as glycerol, lactic acid, citric acid, acetone, and butanol for the rapidly growing chemical industries. Further, the biochemistry of ethanol fermentation was well known over the last 100 years. It is started from Pasteur's research with French wines (in l860s) defined the basic concepts of the fermentation process.

PROPERTIES OF ETHANOL

The **ethanol**, is also known as **ethyl alcohol** or **grain alcohol**, chemical formula is C_2H_5OH, and is abbreviated as EtOH. Pure ethanol is a

flammable, colorless liquid with a boiling point of 78.5° C. Its low melting point of -114.5° C allows it to be used in antifreeze products. Its density is 789 g/l about 20% less than that of water. It is easily soluble in water and is itself a good solvent, used in perfumes, paints and tinctures. Alcoholic drinks have a large variety of tastes, since various flavor compounds are dissolved during brewing. A solution of 70-85% of ethanol is commonly used as a disinfectant; it kills organisms by denaturing their proteins and dissolving their lipids: it is effective against most bacteria and fungi, and many viruses, but is ineffective against bacterial spores. This disinfectant property of ethanol is the reason that alcoholic beverages can be stored for a long time. Ethanol can lose a proton from the hydroxyl group and is a very weak acid, weaker than water. The CAS number of ethanol is 64-17-5 and its UN number is UN 1170. Some of the physical properties of ethanol are given in Table 5.

Table 5: Properties of Ethanol.

Physical state	Clear liquid
Appearance	Colourless
pH	NA
Odor	Mild, pleasant, wine like
Viscosity	1.2 cP at 20oC
Vapor Pressure	59.3 mmHg at 20^{o}C
Boiling Point	78.5^{o}C
Solubility	Miscible
Specific Gravity	0.790 at 20^{o}C
Molecular weight	46.0414
Molecular formula	C_2H_5OH

Ethyl alcohol undergoes a wide range of chemical reactions. Ethanol is used as an important raw material in the chemical industry. Some important reactions are listed below:

(i) Oxidation: Ethanol may be oxidized to acetaldehyde.

$$\underset{\text{Ethanol}}{CH_3CH_2OH} \xrightarrow{O_2} \underset{\text{Acetaldehyde}}{CH_3CHO} + H_2O$$

(ii) Halogenation: Halides of hydrogen, phosphorous and other compounds react with ethanol to replace the – OH group with a halogen:

$$3CH_3CH_2OH + PCl3 \longrightarrow 3CH_3CH_2Cl + P(OH)_3$$

(iii) Reaction with metals: Ethanol reacts with sodium, potassium and calcium to give the
alcoholates (alkoxides) of these metals:

$$2CH_3CH_2OH + 2Na \longrightarrow 2CH_3ONa + H_2$$

(iv) Esters: Ethanol reacts with organic and inorganic acids to give esters:

$$CH_3CH_2OH + HCl \longrightarrow \underset{\text{Ethylchloride}}{CH_3CH_2Cl + H_20}$$

(v) Ethers: Ethanol may be dehydrated to give ethers:

$$2CH_3CH_2OH + CH_3CN_2 \longrightarrow OCH_2CH_3 + H_2O$$

(vii) Alkylation: Addition of alkyl-group with ethanol to form ethylalkylates:

$$2CH_3CH_2OH + H_3SO_4 \longrightarrow \underset{\text{Ethyl hydrogen sulfate}}{CH_3CH_2HSO_4}$$

$$2CH_3CH_2OH + NH_3 \longrightarrow \underset{\text{Ethyl amine}}{CH_3CH_2NH_2}$$

APPLICATIONS OF ETHANOL

1. Solvent use: Ethanol is widely used in industry as a solvent for dyes, oils, waxes, explosives, cosmetics etc.
2. Use as a chemical feed stock: In the chemical industry, ethanol is an intermediate in many chemical processes because of its great reactivity as shown above. It is thus a very important chemical feed stock.
3. General utility: Alcohol is used as a disinfectant in hospitals, for cleaning and lighting in the home, and in the laboratory second only to water as a solvent.
4. Fuel: Ethanol is mixed with petrol or gasoline up to 10% and known as gasohol and used in automobiles.
5. Denatured alcohol: denatured alcohol is usually 95% alcohol with 5% water; for domestic burning or hospital use denatured alcohol is dispened as methylated spirit, which contains a 10% solution of methanol, pyridine and coloring material.

MICROBIAL PRODUCTION OF ETHANOL

When microorganisms grown on sugars in the presence of oxygen, they get energy and cellular carbon material by oxidizing the sugars,

resulting carbon dioxide and water. However, certain microorganisms are able to grow on organic compounds like sugars in the absence of oxygen, partial oxidation of organic compounds end with ethanol and other organic acids. Under anaerobic growth excess production of electrons are accepted by the organic acceptors and produce ethanol as end product. There are several microorganisms including *Saccharomyces cerevisiae, Zymomonas mobilis, Clastridium, E.coli*, etc. Table 6 shows the merits and demerits of some ethanol fermenting organisms.

Table 6: Merits and demerits of ethanol fermentation

Organism	Merits	Demerits
Saccharomyces cerevisiae	• Alcohol yield up to 90% • High tolerance in ethanol (10%) • Naturally adapted to ethanol fermentation	• Not able to ferment xylulose and arabinose • Not able to survive at high temperature of hydrolysis
Zymomonas mobilis	• Compliance to genetic modification • Ethanol yield up to 97% • High ethanol tolerance 14% • Does not require additional oxygen	• Not able to ferment xylulose • Low tolerance to inhibitors
Thermophilic species	• Resistance to high temperature (70^{o}C) • Suitable for bioprocessing	• Production of organic acids • Low tolerance to ethanol

SUBSTRATES FOR ETHANOL FERMENTATION

There is variety of substrates used for ethanol fermentation. Microorganisms utilize diverse organic compounds anaerobically and produce ethanol. The major substrates are from agricultural products including sugar cane, sugar beet, grains, corn, potato and any others. Any fermentable substances rich in glucose or glucose/fructose polymers like cellulose, sucrose, starch and lactose. Cellulose rich waste materials such as straw, timber cuttings, rice hulls, and newspapers, can also be sources of fermentable sugars for ethanol production. Sugar cane and sugar beets contain sucrose (disaccharide), is the best source for industrial ethanol production. In addition, molasses, a byproduct of sugar refining, is also an excellent substrate for ethanol fermentations. For the utilization of cellulose based sugars,

chemical, physical biological pretreatment is required. Through the pre-treatment, simple sugars are released by hydrolysis of complex sugars. When food processing industrial waste is used, certain pretreatment is required, which enrich the fermentable sugar concentration in the raw materials. Fig-13 shows the metabolic process of ethanol fermentation.

FERMENTATION PATHWAY

The primary substrates involved in ethanol fermentations are glucose, sucrose, lactose, and cellulose. All substrates can be metabolized through three basic metabolic pathways. Each substrate will enter into a metabolic pathway is based on appropriate pretreatment process (type of microorganism used). The primary steps involved in the metabolism of these substrates are converted them into catabolic primary metabolism. For example, sucrose is hydrolyzed by the enzyme sucrose phosphorylase to glucose-l-P and fructose. Cellulose is degraded enzymatically in two steps-first to cellobiose (cellulose), then to glucose by cellobiose phosphorylase. Starch, during pretreatment, is hydrolyzed into primarily two fermentable substrates, glucose and maltose. Maltose is then degraded to two glucose molecules. Lactose is metabolized to glucose and galactose which eventually enter the glycolytic pathway.

While most of the substrates used for ethanol production are hexoses. The Embden-Meyerhof pathway is probably the most common pathway for glucose metabolism and proceeds through a series of transformations involving phosphorylation reactions which are important in energy production for growth. The glucose molecule is metabolized to yield two moles of pyruvate per one molecule of glucose/fructose. But, the hexose-monophosphate pathway is slightly different from the Embden-Meyerhof pathway and results in one mole of ethanol and one mole of pyruvate from each mole of glucose or equivalent sugar.

Yeast growing with or without oxygen would metabolize the sugars by the glycolytic pathways. In aerobic processes, compounds reduced in these metabolic steps (NAD+ to NADH) would be reoxidized through an electron transport system with oxygen serving as the final electron acceptor. However, in fermentations oxygen is not present and so the microbe has to use an organic compound as an electron acceptor for

recycling NADH. The pyruvate is an important metabolite act as a branch point for various product formations. In ethanol fermentation, pyruvate is metabolized to acetaldehyde and CO_2. Then acetaldehyde is reduced to ethanol via NADH is reoxidized to NAD+. Each hexose in ethanol fermentation ends with 2 molecules of ethanol, on weight basis, a 51 % conversion of glucose into ethanol. Overall, 90-95% of the theoretical amount of ethanol is produced because certain molecules of pyruvate are consumed for cellular material during culture growth and is not available to serve as an electron acceptor.

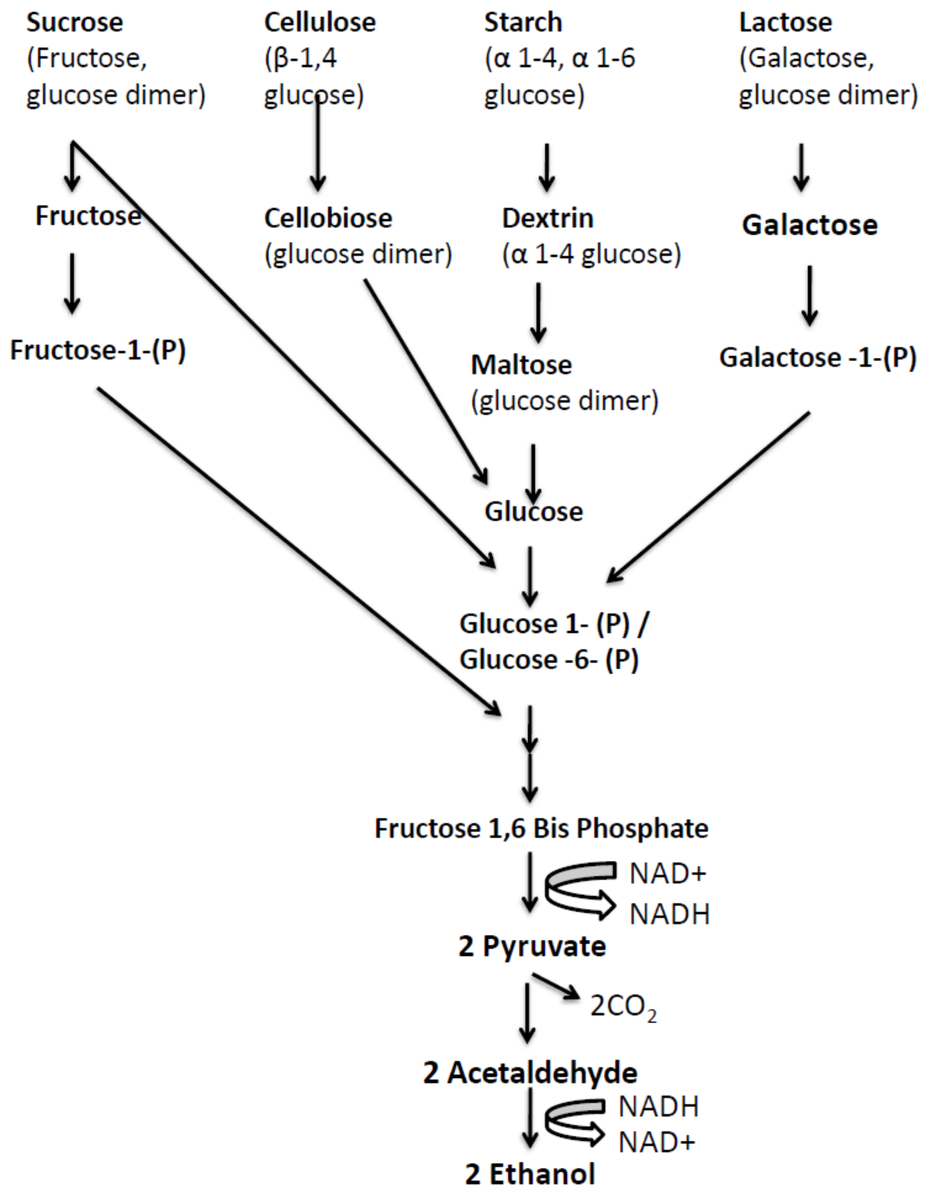


Figure 13 : Metabolic process of ethanol fermentation

The glucose-to-ethanol reaction is represented by the equation below:

$C_6H_{12}O_6 + 2\ ADP + 2\ Pi \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ H_2O + 2\ ATP$

FERMENTATION CONDITIONS

The conditions of fermentation for alcohol production are similar to those already described for whisky or rum production. Alcohol-resistant

yeasts, strains of *Saccharomyces cerevisiae* are used, and nutrients such as nitrogen and phosphate lacking in the broth are added.

Two types of ethanol fermentation process has been proposed, they are i) batch fermentation and, ii) continuous fermentation.

Batch process: Conventional ethanol fermentations are conducted as batch processes where the fermenter is filled with substrate, the microbial inoculum is added, and the fermentation process carried out for 4-10 days. The fermenter is mechanically agitated by impellers to decrease diffusional limitations. In batch processes, the fermentable carbon source is added batch wise. After the fermentation is complete, the cells are removed before distillation.

Continuous process: A modified batch fermenter with continuous-flow operation is used in this method. Here, the sugar and nutrient medium are continually added to the fermenter, and the fermented, which contains ethanol and cell material is continuously treated for product recovery. In this type of fermentation, there is no direct problem of high sugar concentrations adversely affecting cellular growth or ethanol production. The rate of sugar addition has to be regulated so that inhibitory levels of ethanol do not occur and cause decreased growth rates. The process flow diagram is given in fig-14.

Table 7: Nutrients for Ethanol fermentation.

Nutrient	Raw material
Carbon	Molases, starch
Nitrogen	Corn steep liquor, soybean meal, pure ammonia or ammonium salts, urea, nitrate, salts, phosphate salts
Growth Factors	Biotin, yeast extract, beef extract
Minerals	Macro and micro nutrients

Factors for ethanol fermentation

Temperature: Temperature is an important physical parameter influences on the growth rate of the microorganism and ethanol production. Industrial production of wine and beer 20^{o}C is ideal. If the temperature increases above 30^{o}C, ethanol production may decreases.

Table 8 : Parameters for ethanol fermentation.

Parameters	Batch reactor
Volume (Lit)	100
Molases (Kg)	28.2
Initial specific gravity	1.09
Final specific gravity	1.024
Sugar concentration (%)	14.86
Alcohol produced (%)	6.9
Rate of ethanol production	8.07

pH: A very important factor for cellular growth is external pH. Most yeast based ethanol production initial media pH is below 4.5, although this may not be the optimal pH for growth or ethanol production. Yeast cells can grow over a wide pH range from 3 to 8 with an optimum for growth generally in the slightly acidic. Changing pH can also affect the final ratio of organic waste products produced by yeast cultures. Thus, the optimal pH for a fermentation process must support a balance among ethanol production, cellular growth, and physiological effects on waste product pathways.

Oxygen: The alcohol fermentations always in facultative anaerobes. Microbes grow well in the presence of oxygen, when the oxygen is insufficient for oxidative metabolism, it undergoes fermentation process. Hence, for ethanol production, oxygen must be restricted from entering the fermenter and zero-to-very low levels of oxygen in the broth should be maintained.

Ethanol concentration: The concentration of ethanol in the fermentation broth can directly affect the growth of the culture and its ability to convert sugars into ethanol. Inhibitory and toxic levels of ethanol vary from culture to culture. In certain cases, 4-6% ethanol inhibits 40 -50% of yeast. Ethanol tolerant yeast cells survive maximum at 11 – 14% of ethanol.

Fermentable sugar: The concentration of sugar can affect the microbial ethanol fermentation. The amount of alcohol produced is directly related with the amount of sugar present. Usually high sugar concentrations are desired. However, very high concentration of sugar can also inhibits the metabolic activity of cell based on osmotic pressure. In other hand, low level sugar affects the growth as well as the ethanol fermentation. For instance, a mixed substrate is present in the media also yielded low level ethanol.

Other growth requirements: For growth, microbes need balanced nutrients such as nitrogen, phosphorus, potassium, sodium, and sulfur,

as well as trace eements such as zinc, copper, iron, magnesium, and manganese. For ethanol fermentation, yeast required biotin.

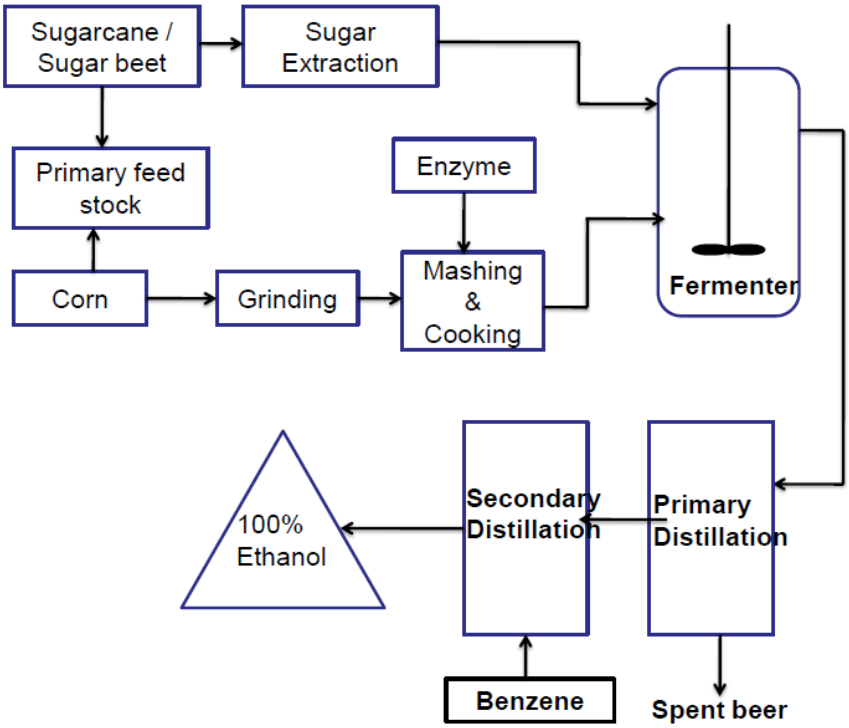


Figure 14: Process flow chart for ethanol fermentation

DISTILLATION OF ETHANOL

After fermentation the fermented liquor contains alcohol as well as low boiling point volatile compounds such as acetaldeydes, esters and the higher boiling, fusel oils. The alcohol is obtained by several operations. First, steam is passed through the beer which is said to be steam-stripped. The result is a dilute alcohol solution which still contains part of the undesirable volatile compounds. Secondly, the dilute alcohol solution is passed into the center of a multi-plate aldehyde column in which the following fractions are separated: esters and aldehydes, fusel oil, water, and an ethanol solution containing about 25% ethanol. Thirdly, the dilute alcohol solution is passed into a rectifying column where a constant boiling mixture, an azeotrope, distils off at 95.6% alcohol concentration.

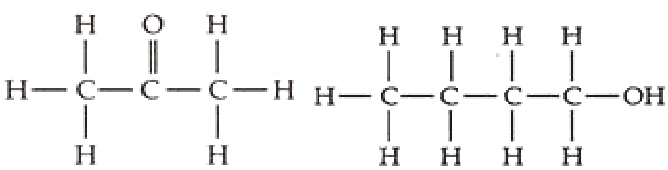
To obtain 200° proof alcohol, such as is used in gasohol blending, the 96.58% alcohol is obtained by azeotropic distillation. The principle of this method is to add an organic solvent which will form a ternary (three-membered) azeotrope with most of the water, but with only a small proportion of the alcohol. Benzene, carbon tetrachloride,

chloroform, and cyclohezane may be used, but in practice, benzene is used. Azeotropes usually have lower boiling point than their individual components and that of benzene-ethanol-water is 64.6°C. On condensation, it separates into two layers. The upper layer, which has about 84% of the condensate, has the following percentage composition: benzene 85%, ethanol 18%, water 1%. The heavier, lower portion, constituting 16% of the condensate, has the following composition: benzene 11%, ethanol 53%, and water 36%. In practice, the condensate is not allowed to separate out, but the arrangement of plates within the columns enable separation of the alcohol. Four columns are usually used. The first and second columns remove aldehydes and fusel oils, respectively, while the last two towers are for the concentration of the alcohol.

13.5.2 ACETONE BUTANOL FERMENTATION

Acetone–butanol–ethanol (ABE) fermentation is a process that uses bacterial fermentation to produce acetone, n-Butanol, and ethanol from carbohydrates such as starch and glucose. It was developed by the chemist Chaim Weizmann and was the primary process used to make acetone during World War I. Acetone was in great demand to manufacture the explosive trinitrotoluene (TNT). Hence, the acetone-butanol fermentation rapidly expanded. But after war, the demand for acetone decreased and butanol increased, as it was required as a solvent for the rapid drying of nitrocellulose paints in automobile industry. Thus, the commercial process of acetone-butanol survived even after a lack of demand of acetone after World War-I.

Biochemistry of fermentation



Acetone, C_3H_6O n Butanol, $C_4H_{10}O$

In a normal batch culture, solvent-producing Clostridium species produce hydrogen, carbon dioxide, acetate, and butyrate during the initial growth phase (acidogenic phase), which results in a decrease in the pH of the culture medium.

As the culture enters the stationary growth phase, the metabolism of the cells undergoes a shift to solvent production (solventogenic phase).

During the second phase of the fermentation the reassimilation of acids, which occurs concomitantly with the continued consumption of carbohydrate, normally results in an increase in the pH of the culture medium.

Hexose sugars (including mono-, di-, tri-, and polysaccharides) are metabolized via the Embden-Meyerhof pathway with the conversion of 1 mol of hexose to 2 mol of pyruvate, with the net production of 2 mol of adenosine triphosphate (ATP) and 2 mol of reduced NADH. The solvent-producing clostridia metabolize pentose sugars by way of the pentose phosphate pathway. The pentoses fermented are converted to pentose 5-phosphate and dissimilated by means of the transketolase-transaldolase sequence, resulting in the production of fructose 6-phosphate and glyceraldehyde 3-phosphate, which enter the glycolytic pathway. The fermentation of 3 mol of pentose yields 5 mol of ATP and 5 mol of NADH.

The pyruvate resulting from glycolysis is cleaved by pyruvate ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to yield carbon dioxide, acetyl-CoA, and reduced ferredoxin. Acetyl-CoA produced by the phosphoroclastic cleavage is the central intermediate in the branched fermentation pathways leading to both acid and solvent production.

During acid-producing metabolism there is a rapid flow of electrons derived both from the phosphoroclastic cleavage of pyruvate and from NADH through ferredoxin to produce molecular hydrogen. A separate enzyme, NADPH ferredoxin oxidoreductase, apparently functions in the controlled production of NADPH from reduced ferredoxin, which is required for biosynthesis.

Acid-Producing Pathways

In acid production the phosporoclastic cleavage of pyruvate to acetyl-CoA, C. acetobutylicum can also convert pyruvate to lactate under certain conditions. The lactic acid pathway is not operational under normal conditions, and this pathway only appears to operate as a less efficient alternative to allow energy generation and the oxidation of NADH to continue when the mechanisms for the disposal of protons and electrons by the generation of molecular hydrogen is blocked.

The carbon flow from acetyl-CoA through the main branches of the pathway leading to the formation of acids and solvents is shown in Fig.

15. These branch points arise from three key intermediates, acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA. During the acid-producing phase, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA by means of two analogous steps which result in the production of the corresponding acyl-phosphate, followed by the generation of ATP. The four enzymes involved in the metabolic pathway responsible for the formation of butyryl-CoA from acetyl- CoA are thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase.

Solvent-Producing Pathways

During solvent production, acetyl-CoA and butyryl-CoA function as the key intermediates for ethanol and butanol production. These pathways produce acetylaldehyde and butyraldehyde, respectively, as intermediates, and the pathway requires the function of two sets of dehydrogenases to accomplish the necessary reductions to produce ethanol and butanol.

The reduction of butyryl-CoA to butanol is mediated by butyraldehyde dehydrogenase and butanol dehydrogenase. In both *C. acetobutylicum* and *C .beijerinckii* the activity of butanol dehydrogenase has been reported to be NADPH dependent rather than NADH dependent. Although the analogous acetaldehyde dehydrogenase and ethanol dehydrogenase have not been identified as being enzymes separate from those involved in butanol production, this seems likely as ethanol can be produced independently from acetone and butanol by C. acetobutylicum under certain culture conditions.

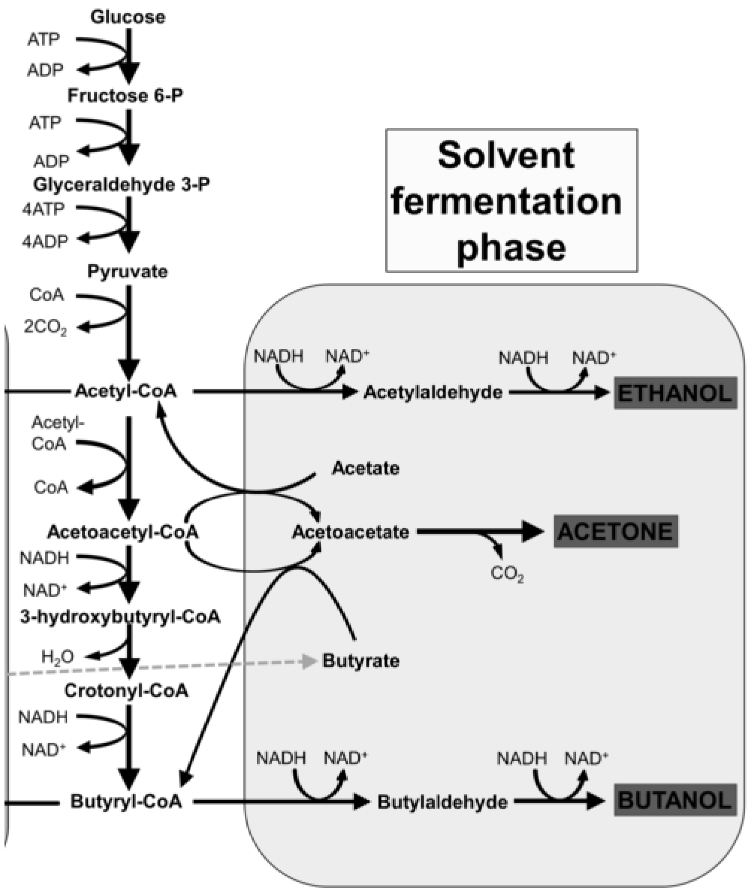


Figure 15: Pathway of Acetone butanol Fermentation.

The organism *Clostridium acetobutylicum* will ferment a variety of carbohydrates such as lac-tose, glucose, xylose, fructose, arabinose, galactose, maltose, man'nose, starch and sucrose (Buchanan and Gibbons, 1974), and produce a variety of organic solvents including butyric and acetic acids, butanol, acetone, ethanol, carbon dioxide, hydrogen, isopropanol, formic acid, ace-tone (acetylmethylcarbinol or 3-hydroxy-2-butanone) and a yellow oil, which is accomplex mix-ture of higher alcohols, higher acids and esters. Butanol, acetone and ethanol are normally considered to be the principal products of this fermentation.

FERMENTATION PROCESS OF ACETONE-BUTANOL:

(i) Production of Inoculum: Two species of Clostridium, which differ slightly in their nutritional requirements and fermentation factors, are generally employed for acetone-butanol fermentation.

Clostridium acetobutylicum and *Cl. saccharo-acetobutylicum* are the species involved. The fermentation by the former requires corn medium and the later molasses medium for the growth. In general, inoculum growth and fermentative production of the solvents are carried out at 31° to 32°C for *Cl. saccharoacetobutylicum* and at approximately 37°C for *Cl. acetobutylicum*.

(a) Inoculum of *Cl. saccharoacetobutylicum*: Inoculum of *Cl. saccharo acetobutylicum* is developed employing molasses, calcium carbonate, ammonium sulphate or phosphate and sometimes corn-steep liquor. Clostridia, being spore formers, are easily maintained as soil stocks in contrast to the vegetative cells, the spores are not very sensitive to oxygen. However, prolonged storage of these spores leads to decrease in the acetone butanol production.

RAW METERIALS FOR MEDIA FORMULATION

Starch: The production of acetone and butanol by the Weizmann process utilized starch as substrate (Beech, 1953). C.acetobutylicum (Weizmann) possessed amylolytic and saccharolytic enzyme activities required to hydrolyze gelatinized starch to glucose and maltose. Concentrations of corn of 8 to 10% (5 to 6.5% starch) were readily utilized; average solvent yields of 38.0%, based on sugar fermented were reported. Wheat, milo and rye served equally well.

Molasses: Beech (1952) described the use of a variety of other Clostridium species used for fermentation of sugar in molasses. The fermentation process was similar to that described above for starch except that the mash could be sterifized'at a lower temperature, the fermentation was conducted at 31 to 32°C, higher" concentrations of sugars could be used and a greater ratio of butanol to acetone plus ethanol was produced. It was necessary to add nitrogen and phosphate nutrients to molasses fermentations(1.0 to 1.4% ammonia based on sugar present).

Lignocelluosit Hydrolysates: *C. acetobutylicum* can anaerobically ferment different carbohydrates into 'a spectrum of Products including n-butanol, acetone, ethanol, butyric acid, acetic acid and acetoin. Hydrolysates of lignocellulosics wood, paper, crop residues, ete. , primarily contain glucose and cellobiose as well as galactose, and the pentose sugars. Glucose, mannose, arabinose are efficiently utilized by this organism and produce acetone, butanol and ethanol.

Fermentation phases

(a) First Phase: In this phase rapid growth of the bacterium and formation of acetic acid and butyric acid in large amounts along with the production of large quantities of carbon dioxide and hydrogen gases. The pH of the medium which was initially 5.0 to 6.5 for corn medium and 5.5 to 6.5 for molasses medium, decreases and then remains constant for the rest of the fermentation process. This phase, lasts for approximately 13 to 17 hours of incubation. The titratable acidity increases to a maximum and adaptive enzymes are produced which convert acids to neutral solvents.

(b) Second Phase: A sharp decrease in the titratable acidity due to conversion of more acids into acetone and butanol. This process is called as acid break, which gets delayed if there is contamination. The rate of gas formation reaches maximum after acid break. However, it gradually slows as the fermentation process proceeds further.

(c) Third Phase: The rate of gas formation decreases substantially along with decreased rate of solvent production. The titratable acidity slowly increases leading to a pH of 4.2 to 4.4 in the corn medium and 5.2 to 6.2 in the molasses medium. Many cells undergo autolysis at this point resulting in the release of riboflavin into the medium.

Yield: The ratio of yield of acetone, butanol and ethanol differ slightly depending on the fermentation medium. But, generally the yield is 2% by weight of the broth, which is approximately equal to 30% conversion of carbohydrate to solvents. In a corn medium the ratio of butanol, acetone and ethanol are 6:3:1 respectively, but in molasses medium the ratios are 6:5:3.

The acetone-butanol fermentation yields several products in addition to the gases (CO_2). They include isopropanol, formic acid, acetic acid, butyric acid, acetylmethyl carbinol and yellow oil, which is a mixture of higher alcohols and acids, which are industrially very important. Contamination due to bacteriophages and Lactobacillus is a common problem which can be prevented by undertaking absolute sterilization. The entire process is illustrated in fig.16

Product Recovery: A beer still is used for the recovery of the products from the fermentation broth. The beer still is a tall vertical and continuous still consisting of about 30 perforated plates. The recovery process consists of the following steps:

1. The fermentation broth is allowed to enter the still from top. It descends the still passing through perforated plates.
2. A continuous flow of steam is allowed into the still from its bottom. It moves up the still in a direction opposite to the direction of fermentation broth.
3. Acetone and butanol vaporizes due to the effect of steam.
4. The steam and solvents are then collected and condensed by cooling to get a solution which contains approximately 40% by weight of solvent mixture.
5. The individual solvents present in the solvent mixture are separated by fractional distillation.
6. Acetone and butanol are collected in separate fractions.
7. Ethanol and isopropanol are also collected as a single fraction and sold as a general solvent.
8. The residue contains riboflavin and other B vitamins as well as considerable quantity of bacterial cells. The residue is concentrated and dried and used as vitamin feed supplement. Flow diagram for the production of acetone butanol is shown in the Fig. 16.

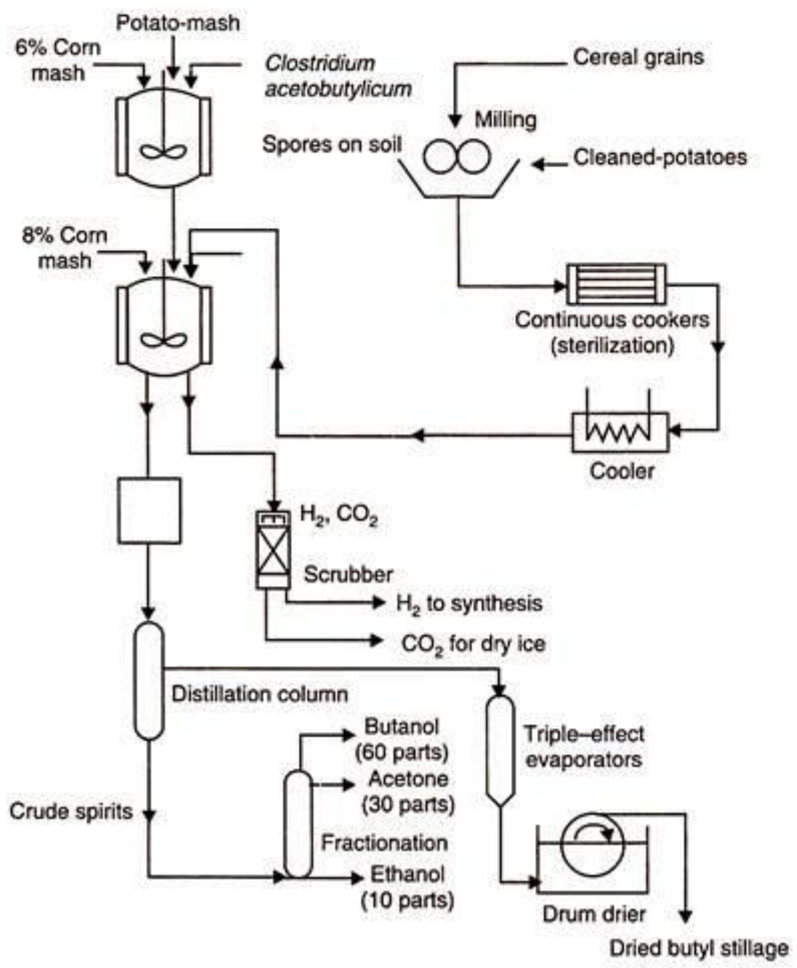


Figure 16 – Flow diagram of acetone –butanol fermentation

USES OF ACETONE-BUTANOL

1. At present butanol is extensively used in brake fluid antibiotic recovery procedures, urea, formaldehyde resins, amines for gasoline additives and as ester in the protective coating industry.

2. Butanol is also used for the synthesis of butadiene which is used in the preparation of synthetic rubber.

3. Acetone is used as a universal organic solvent and also in the preparation of explosives like trinitrotoluene.

Check your Progress -2
Note: Write your answer in the space given below
c. Give the applications of ethanol.
d How acetone-butanol fermentation carried out?
……………………………………………………………………………

12.6 LET US SUM UP

In this chapter, you leant about the commercial importance of organic acids (citric acid and Lactic acid), amino acids (L-Glutamic acid and Lysine), and solvents (ethanol and acetone). The organism involved, metabolic pathways involved, carbon and nitrogen requirements of the organism, the microbial metabolism and biochemistry of the desirable end products such as organic acids, amino acids and solvents. Further, recovery of products from the fermented media also discussed elaborately. Applications of the solvents, organic acids and aminoacids are also discussed.

12.7 UNIT - END EXERCISES

1. How ethanol is fermented in industries?
2. Discuss about acetone butanol fermentation.
3. Explain the fermentation process of citric acid.

12.8 ANSWERS TO CHECK YOUR PROGRESS

a) *Aspergillus niger* is the primary production of citric acid.
b) Lactic acid used as a acidulant for vegetables, leather processing industries, textile processing, electroplating and detergent making.
c) Ethanol is used as a solvent, chemical fuel stock, general purpose use, fuel and used on disinfectant, etc.,
d) Acetone butanol fermentation is carried out in three phase: submerged fermentation process by *Cl. Acetobutylicum.*

12.9 SUGGESTED READINGS

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NOTES

UNIT -XIII: MICROBIAL PRODUCTION OF ANTIBIOTICS AND VITAMINS

Structure

13.1 INTRODUCTION

A chemical compound acting against the life is called an antibiotics. It can be obtained either from natural sources or by a synthetic method. Microbes are the best source of antibiotic production. In this regard, microbes can also produce several value added products including the vitamins. Vitamins such as B12, and riboflavin are the best examples for industrially producing vitamins have larger commercial value. In this chapter, microbial production of antibiotics such as Penecillin and Streptomycine as well as the vitamins such as B12 and riboflavin are discussed.

13.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the process of penicillin production
- Understand the method of streptomycine production.
- Know the microbial production and recovery of vitamin B12.
- Study the bacteriological production of riboflavin.

13.3 MICROBIAL PRODUCTION OF ANTIBIOTICS

Antibiotics are non-growth associated, secondary metabolites produced at the stationary phase growth of microbes. They are otherwise called as chemotherapeutic agent. The terms ‘antibiotic’ and ‘antibiosis’ were used in 20th century when Alexander Fleming in 1930s accidentally found a mould as a contaminated culture on Petri dish or plate agar while he was cultivating microorganisms. Fleming

first identified the drug penicillin, which was isolated from *Penicillium notatum*. The antibiotic discovery made by Fleming had great impact in the antimicrobial therapy, and he was awarded the Nobel Prize, in the field of medicine and physiology in 1945. More than 100s of antibiotics produced by microorganisms are in commercial use.

The commercial production of penicillin and other antibiotics is the most dramatic example of industrial microbiology. The annual production of bulk penicillin is about 33 million pounds. Several microorganisms including bacteria, actinomycetes, fungi and algae produce many most important antibiotics. Among the various antibiotic producers, Bacillus from the bacteria, Streptomyces from the actinobacteria and Penecillium from the fungi are notable genera produce multiple antibiotics. In this chapter, microbial production of Penecillin and Streptomycin antibiotics are given below.

14.4 PENICILLIN

Penicillin was first discovered in 1928 and is now the most widely used antibiotic in the world. The invention started with the developed mold on a staphylococcus culture plate. In 1928, Sir Alexander Fleming, a Scottish researcher, is acknowledged with the discovery of penicillin. During that time, Fleming was working and experimenting with the influenza virus in the Laboratory at St. Mary's Hospital in London. After two weeks, he observed that a mold developed accidentally and contaminated the staphylococcus culture plate. He examined the mold and noticed that the presence of culture prevented staphylococci growth.

Penicillin is a secondary metabolite of fungal species, which is used as an antibiotic by inhibiting the synthesis of peptidoglycan membrane in the gram-positive bacteria, resulting in cell lysis.

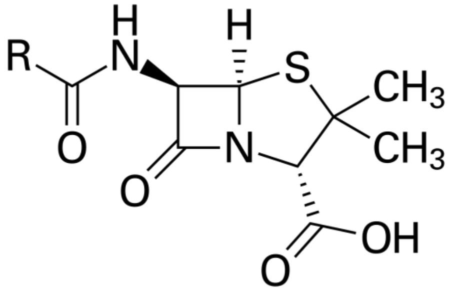


Figure-1. Basic structure of Penicillin core, where "R" is the variable group.

The core has the molecular formula R-$C_9H_{11}N_2O_4S$, where R is the variable side chain that differentiates the penicillins from one another. The penam core has a molar mass of 243 g/mol, with larger penicillins having molar mass near 450—for example, cloxacillin has a molar mass of 436 g/mol. The key structural feature of the penicillins is the four-membered β-lactam ring; this structural moiety is essential for penicillin's antibacterial activity. The β-lactam ring is itself fused to a five-membered thiazolidine ring. The fusion of these two rings causes the β-lactam ring to be more reactive than monocyclic β-lactams because the two fused rings distort the β-lactam amide bond and therefore remove the resonance stabilisation normally found in these chemical bonds.

Penicillin is not a single compound, but a group of chemical compounds with related structure and activity. There are six types of penicillins namely, Penicillin G (benzyl penicillin), F, X, V, K and O. Of the large number of several antibiotics, penicillin still represents the key point in the discovery of effective antibiotics, a milestone in therapeutic medicine. The invention of penicillin modified the course of medicine and has allowed the physicians to treat life-threatening and severe illnesses such as bacterial meningitis, endocarditis, pneumococcal pneumonia, syphilis and gonorrhea.

Mechanism of action: Bacteria constantly remodel their peptidoglycan cell walls, simultaneously building and breaking down portions of the cell wall as they grow and divide. β-Lactam antibiotics inhibit the formation of peptidoglycan cross-links in the bacterial cell wall. The four-membered β-lactam ring of the penicillin binds to the enzyme DD-transpeptidase. As a result, DD-transpeptidase cannot make cross-links, and an imbalance between cell wall production and degradation develops, causing the cell to rapidly die.

BIOSYNTHESIS OF PENICILLIN

The β-lactum thiazolidine ring of penicillin is formed by the condensation of L-cystine and L-valine. The biosynthesis occurs in a non-ribosomal process by means of dipeptide composed of alpha aminoadipate (α – α – AAA) and α-cystine or a breakdown product of cystothiamine. Subsequently L-valine is connected via epimerization reaction resulting in the formation of tripeptide. The first product of cyclization of the tripeptide which can be isolated is isopenicillin N but the biochemical reactions leading to this intermediate is not understood.

Benzyl penicillin is produced in exchange of α -α-AAA with activated phenylacetic acid (Fig.2).

About 38% of the penicillins produced commercially are used as human medicine, 12% in veterinary medicine and 43% as starting materials for the production of semi-synthetic penicillins.

FERMENTATION OF THE PRODUCT

Microorganisms

Semi-synthetic penicillin was first developed in the late 50's with the production of benzyl-penicillin commercially, in which the fermentation process is highly induced in the Penicillium mold by adding Phenyl acetic acid ($C_6H_5.CH_2.COOH$). Since then large scale of production of penicillin production has been developed. The yield of penicillin was regularly monitored and many changes to the extraction and fermentation stages have occurred in the last 50 years to optimize the penicillin production. The improvement in the production of penicillin started in 1943 with the help of *Penicillium chrysogenum* strain NRRL 1951 and is best suited for the submerged production of penicillin than the original strain *Penicillium notatum.* The complete flow diagram of the industrial production of penicillin is given in Fig. 2.

Media used

- For inoculum medium - Calcium carbonate (3.5%), glucose (1.0%), phenyl acetic acid (0.5%), cornsteep liquor (3.5%), and sodium hydrogen phosphate (0.4%), is used.
- Antifoaming agent (0.25%) – silicone, lard oil and octadecanol are added to prevent foaming during the fermentation process.

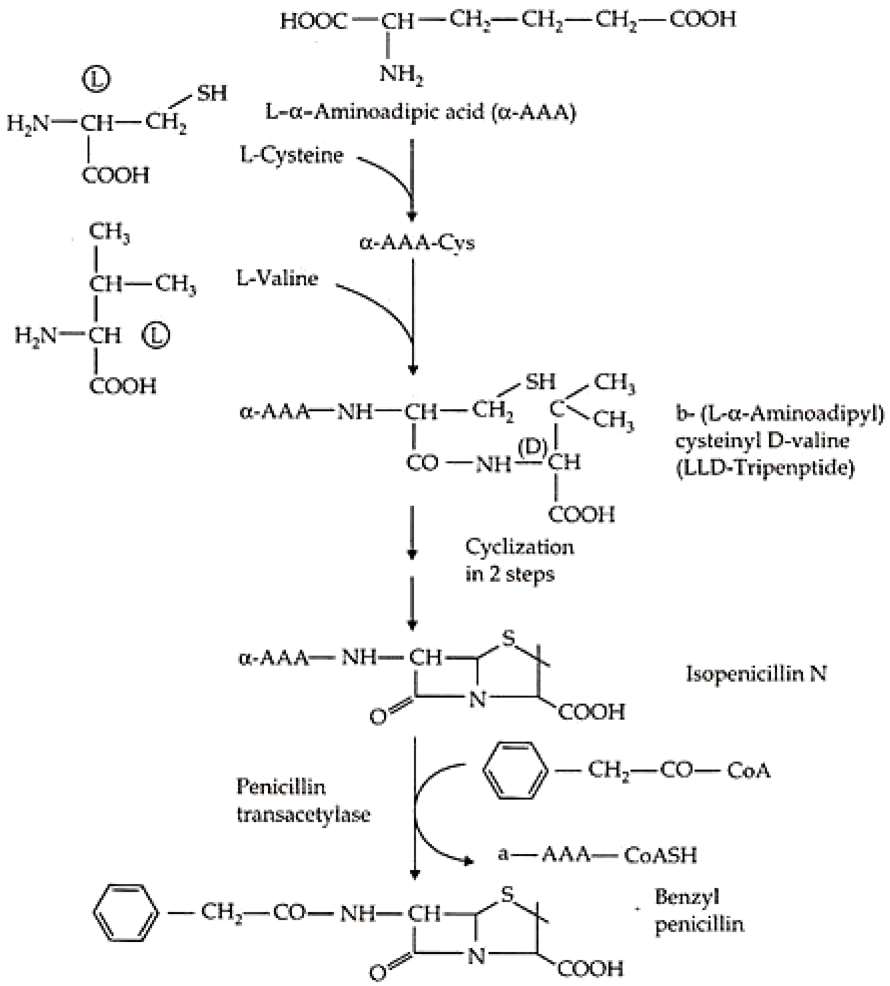


Figure 2: Biosynthesis of penicillin.

Industrial production

- Inoculate the spores of *P.chrysogenum* in a 500 mL flask and incubate on a rotary flask at 25^0 C at 250 rpm for 1 week.
- Then the culture will be transferred to big inoculum tanks and incubate for 1-2 days under good aeration, this helps in aiding mycelia growth. Then the contents are transferred to big fermentors containing the production medium (listed in the media composition) and maintained at 25-26^0 C and pH in the range of 6.5.
- The process is carried out under aerobic condition with the regular feeding (fed-batch process). The growth of fungus in the submerged form is mostly as mycelial cells. The process is carried out for 7 days. Initially, carbohydrates are completely used up and the mycelial growth occurs in the medium. This

starts to reduce the level of carbohydrate content in the medium; thus it favours the production of penicillin, which starts from the second day of fermentation process. Automatically, the pH of the medium increases to 8.0 by the 7^{th} day, and the penicillin production stops at this day.

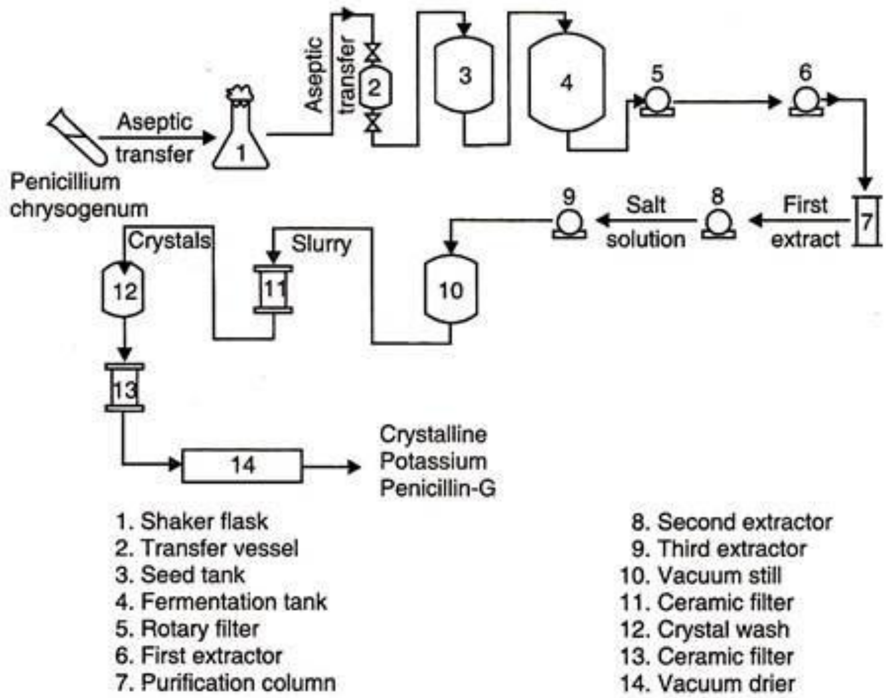


Figure 3: Flow chart for large scale production of Penicillin.

RECOVERY AND PURIFICATION OF PENICILLIN

After sufficient amount of penicillin has been produced during fermentation process, it is extracted and then purified. The entire process is carried out in three different stages.

1. Separation of mycelium
2. Extraction of penicillin and
3. Treatment of crude extract

The treatment of crude extract is as follows; (Fig-4)

1. Extraction of penicillin
2. Treatment with carbon (charcoal)
3. Transfer to aqueous phase
4. Recovery of solvents
5. Crystallization

6. Crystal washing
7. Crystal drying

Harvest broth from fermenter

↓

Chill to 5-10^0 C

↓

Filter of *P.chrysogenum* mycelium rotary vaccum filter

↓

Acidify filtrate to pH 2.0 – 2.5 with H_2SO_4

↓

Extract penicillin from aqueous filtrate into butyl acetate in a centrifugal counter current extractor (treat/dispose aqueous phase)

↓

Extract penicillin from butylacetate into aqueous buffer (pH 7.0) in a centrifugal counter current extractor (recover and recycle butyl acetate)

↓

Acidify the aqueous fraction to pH 2.0-2.5 with H_2SO_4 and reextract penicillin into butyl acetate as in stage 5

↓

Add potassium acetate to the organic extract in a crystallization tank to crystallize the penicillin as a potassium salt

↓

Recover crystals in a filter centrifuge (recover and recycle butyl acetate)

↓

Further processing of penicillin salt

Figure 4: Steps involved in penicillin purification.

At harvest the broth is filtered on a vacuum filter inorder to remove the mycelium and other materials. Sulphuric acid or phosphoric acids are added to decrease the pH level (2 to 2.5) in order to change the penicillin to the anionic form. Then the broth is extracted in a Solvent extractor with the addition of organic solvents like butyl acetate or amyl acetate and isobutyl ketone. Rapidly proceed this step because penicillin is highly unstable at low pH ranges. Penicillin is then extracted into water from the solvents by adding an enough amount of sodium or potassium hydroxide to form penicillin salt. Then the aqueous solution is acidified and re-extracted with methyl isobutyl ketone. Then the solvent extract is completely extracted with NaOH and cause the penicillin to crystallize as potassium or sodium penicillinate. The final product of penicillin salts are washed and air dried. Sometimes the impurities and pigments are removed from the crude extract of penilllin by treatment with charcoal. Then the final crystal product is absolutely 99% pure penicillin. Finally, the antibiotic is packed in a sterile vial as a suspension or a powder.

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14.3.2 STREPTOMYCIN

Streptomycin, produced by the actinomycetes *Streptomyces griseus* is active against Gram (-) ve bacteria and against tuberculosis bacterium, *Mycobacterium tuberculosis*. However, it proved to be useful in the treatment of infections caused by Gram (+) ve specially resistant to penicillin. It is also useful in the control of plant diseases caused by bacteria as it acts systemically in plants. One of the disadvantages of streptomycin is its neurotoxicity due to which hearing impairment and balance maintenance is lost in man due to prolonged streptomycin treatment at high dosage. Its reduction to dihydrostreptomycin results in the decreased toxicity. For this reason in recent times only dihydrostreptomycin is being produced due to ready development of resistance against streptomycin. It is used mostly in conjunction with para aminosalicyclic acid or isoniazid (isonicotinic acid hydrazide) which minimizes resistance build up in sensitive microorganisms.

CHEMICAL STRUCTURE OF STREPTOMYCIN

Streptomycin and dihydrostreptomycin is an aminoglycoside antibiotic and basic compound which is available as hydrochloride,

$C_{21}H_{39}N_7O_{12}$. 3 HCl, as a crystalline hydrochloride double salt with calcium chloride or as phosphate or sulphate and dihydrostreptomycin as the hydrochloride or sulfate. The chemical structure of streptomycin is given in Fig. 5. Streptomycin can be used clinically to treat tuberculosis in combination with other medications and susceptible strains which cause bacterial endocarditis.

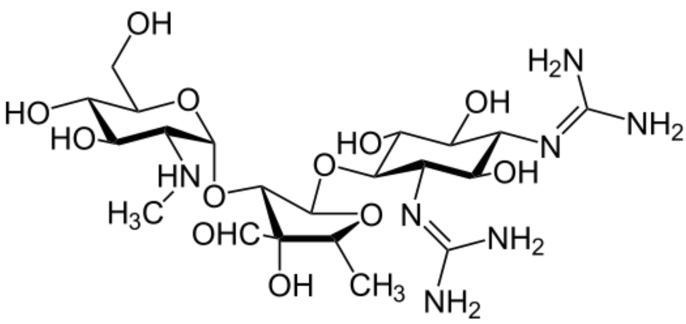


Figure 5: Structure of streptomycin.

Unit of streptomycin activity is equal to one microgram of the free base. Use of precursor does not increase yields of streptomycin.

Mechanism of action: Streptomycin is a protein synthesis inhibitor. It binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. This leads to codon misreading, eventual inhibition of protein synthesis and ultimately death of microbial cells through mechanisms that are still not understood. At low concentrations, however, streptomycin only inhibits growth of the bacteria by inducing prokaryotic ribosomes to misread mRNA. Streptomycin is an antibiotic that inhibits both Gram-positive and Gram-negative bacteria, and is therefore a useful broad-spectrum antibiotic.

BIOSYNTHESIS OF STREPTOMYCIN

Streptomycin is directly derived from glucose. Though the enzymes involved in the synthesis of N-methyl glucosamine are not yet known, it is expected that about 28 enzymes take part in the conversion of glucose into streptomycin as precised in Fig. 6. Glucose 6-phosphate obtained from glucose takes three independent routes to respectively produce streptidine 6-phosphate, L-dehydrostreptose and N- methyl glucosamine. The former two compounds condense to form an intermediate which later combines with methyl glucosamine to produce

di-hydro-streptomycin-6-phosphate. This compound, in the next of couple of reactions, gets converted to streptomycin. The nutrient sources-carbohydrates (glucose), ammonia and phosphate also regulate (by feedback mechanism) streptomycin production.

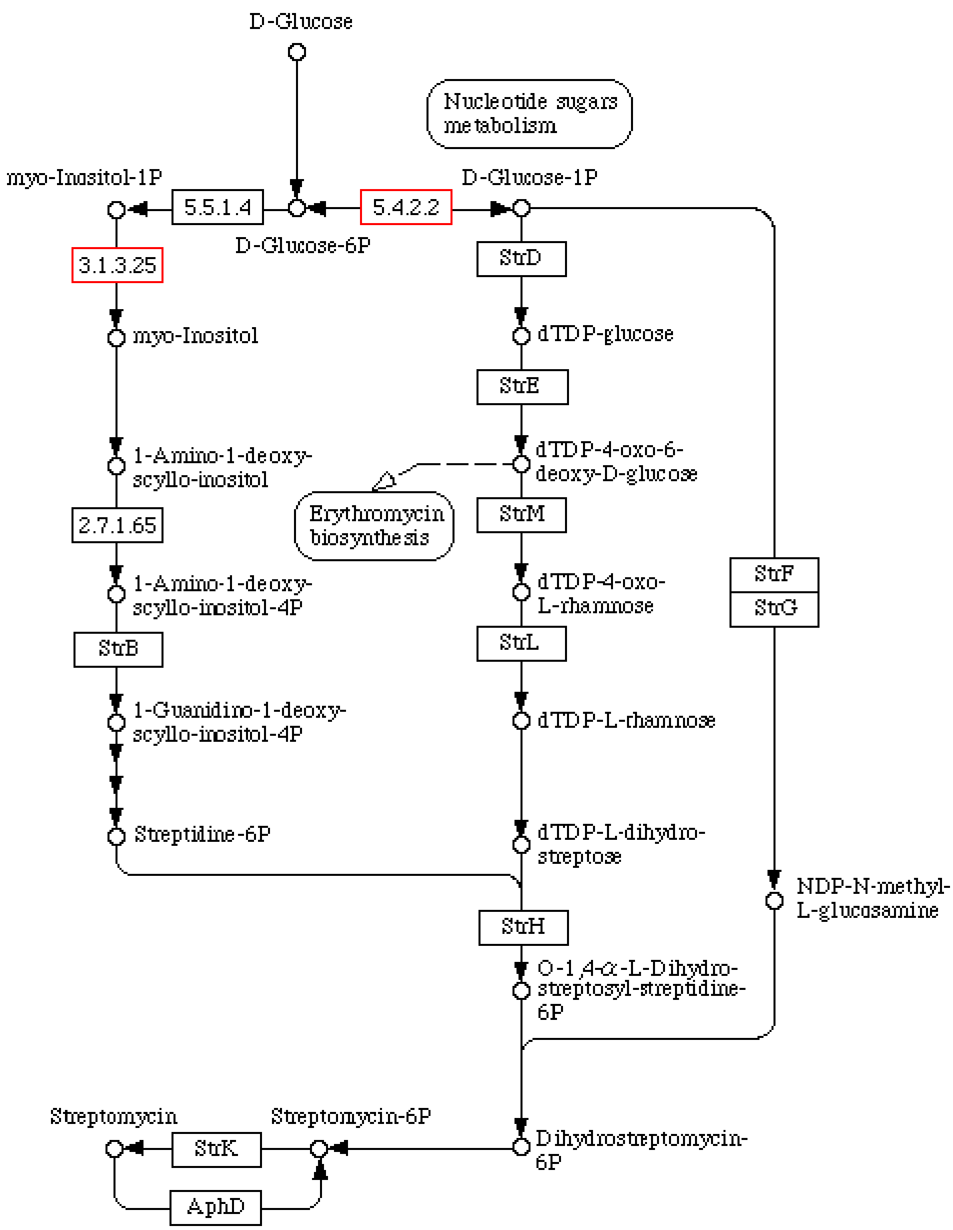
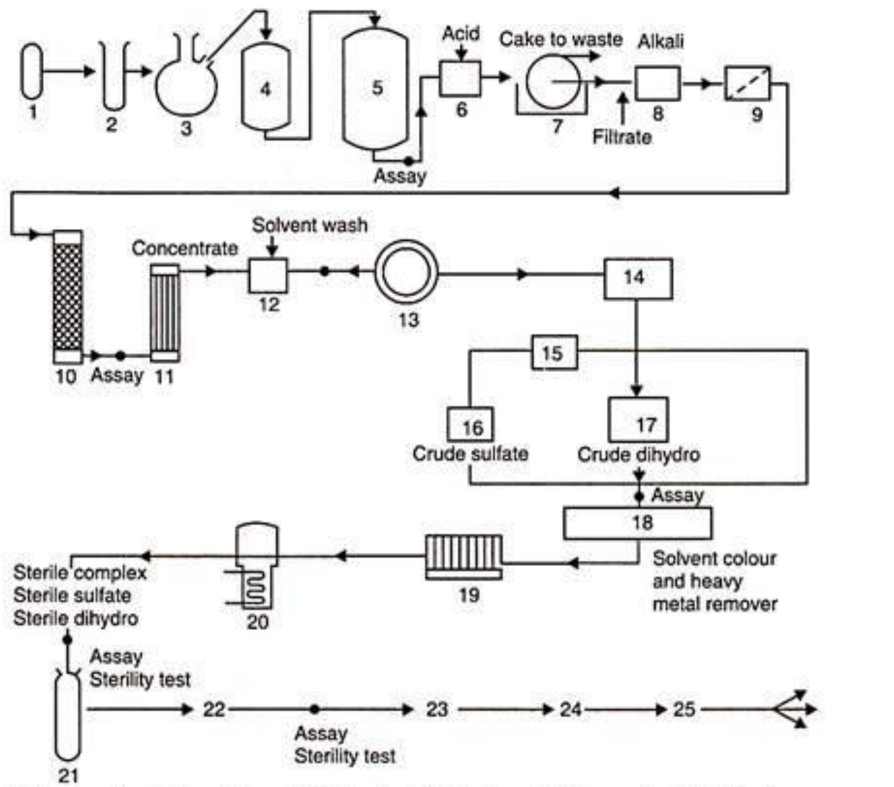


Figure-6: Biosynthesis of streptomycin.

FERMENTATION PROCESS OF STREPTOMYCIN

Industrially streptomycin is produced by submerged culture method, whose flow sheet is given in Fig. 7. When Woodruff and Mc Daniel (1954) suggested medium consisting of soyabean meal (1%), glucose 1% and sodium chloride (0.5%), Hocken hull (1963) recommended the medium consisting of glucose (2.5%), soyabean meal (4.0%), distillers dry soluble (0.5%) and sodium chloride (0.25%) and pH 7.3-7.5 for production of streptomycin by *S. griseus*.



1. Master culture 2. Agar slopes 3. Shaker flask 4. Seed vessel 5. Fermentor 6. Acidification
7. Filtration 8. Neutralization 9. Filter clarification 10. Ion-exchange reagent 11. Evaporator
12. Crystallization 13. Vacuum oven 14. Calcium chloride crude complex 15. Calcium chloride removal
16. Crystallization 17. Catalytic hydrogenation 18. Finishing 19. Seitz filter 20. Freeze drying
21. Vial filling machine 22. Capping 23. Labelling 24. Packing 25. Despatch

Figure-7: Flow diagram of Streptomycin fermentation.

(i) The Inoculum Production: Spores of *S. griseus* maintained as soil stocks or lyophilized in a carrier such as sterile skimmed milk, is employed as stock culture. The spores from these stock cultures are then transferred to a sporulation medium to provide enough sporulated growth to initiate liquid culture build-up of mycelial inoculum in flasks

or inoculum tanks. After sufficient mycelial growth, it is fed to production fermenter.

(ii) Preparation of the Medium: A production medium contains carbon source and nitrogen source. Glucose is one of the best carbon sources which helps in the greater yield of streptomycin, because it provides basic carbon skeleton for the streptomycin production. Apart from glucose, fructose, maltose, lactose, galactose, mannitol, xylose and starch can also be used as carbon source. Polysaccharides and oligosaccharides generally give low yields.

Peptones, soya extracts, meat extract, the residue from alcohol distillation, ammonium salts, nitrates and glycine may be used as nitrogen source. Magnesium, calcium, potassium, boron and molybdenum may be used as mineral source along with sulphates, phosphates and chlorides.

Phenylacetic acid, L-naphthalene acetic acid may be added as growth stimulating compounds. It is better to add proline into the medium which helps in high streptomycin production. Fats, oils and fatty acids may also be used along with glucose. If necessary antioxidants such as sodium sulphate or starch or agar may also be added into the medium. There is no need of precursor in the production of streptomycin.

(iii) Fermentation conditon: Sterilized liquid medium with all the above substances is fed to the production fermenter. Appropriate volume of inoculum (4-5%) is introduced into it. The optimum fermentation temperature is in the range of 25 to 30°C and the optimum pH range is between 7.0 and 8.0. High rate of streptomycin production, however, occurs in the pH range of 7.6 to 8.0. The process of fermentation is highly aerobic and lasts approximately for 5 to 7 days and passes through 3 phases:

1. **The First Phase:** It takes about 24 hours to 48 hours. Rapid growth and formation of abundant mycelium occurs during this phase. The pH rises to 8.0 due to release of ammonia into medium, due to proteolytic activity of S. griseus. Glucose is utilized slowly and little production of streptomycin is witnessed.
2. **The Second Phase:** It lasts for 2 days. Streptomycin production takes place at a rapid rate without increase in the mycelial

growth. The ammonia released in the first phase is utilized, which results in the decrease of pH to 7.6-8.0. Glucose and oxygen are required in large quantity during this phase.

3. **Third Phase:** Cells undergo lysis, releasing ammonia and increase in the pH, which falls again after a period of continuous streptomycin production. Requirement of oxygen decreases and the contents of the medium including sugar get exhausted. Finally streptomycin production ceases. A yield of 1200 micrograms per milliliter of streptomycin is obtained.

RECOVERY AND PURIFICATION OF STREPTOMYCIN

After fermentation the mycelium is separated from the broth by filtration. Streptomycin is recovered by several methods.

The fermentation broth is acidified, filtered and neutralized. It is then passed through a column containing a cation exchange resin in an ion-exchange column to adsorb the streptomycin from the broth. The column is then washed with water and the antibiotic is eluted with hydrochloric acid or cyclohexanol or phosphoric acid. It is then concentrated at about 60°C under vacuum. The streptomycin is then dissolved in methanol and filtered and acetone is added to the filtrate to precipitate the antibiotic. Treatment with activated carbon is often necessary to remove impurities. Streptomycin can be precipitated in the form of sulfate salt. The precipitate is again washed with acetone and vacuum dried. It is purified further by dissolving in methanol. The streptomycin in pure form is extracted as calcium chloride complex.

Check your Progress-1
Note: Write your answer in the space given below
a. Discus the mechanism of action of penicillin.
b. How streptomycin is produced?.
…………………………………………………………………………
…………………………………………………………………………
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13.4 MICROBIAL PRODUCTION OF VITAMINS

Vitamins are organic compounds that catalysis specific biological functions for normal function and optimal growth of an organism. These vitamins cannot be synthesized by the higher

organisms, including human being, and therefore they have to be supplied in small amounts in the diet. Microorganisms are capable of synthesizing the vitamins. In fact, the bacteria in the gut of humans can produce some of the vitamins, which if appropriately absorbed can partially meet the body's requirements. It is an accepted fact that after administration of strong antibiotics to humans (which kill bacteria in gut), additional consumption of vitamins is recommended. In this chapter, microbial production of vitamin B12 and riboflavin are discussed.

13.4.1 VITAMIN B12

Cyanocobalamin or B12 is an important group of water-soluble compounds belonging to the cobalt corrinoid family. The naturally occurring derivatives are hydroxycobalamin (OHB_{12}), adenosylcobalamin ($AdoB_{12}$), and methylcobalamin (MeB_{12}). The empirical formula of cyanocobalamin is $C_{63}H_{88}CoN_{14}O_{14}P$. The structure of vitamin B12 has corrin ring with a central cobalt atom. The corrin ring is similar to the structure of tetrapyrrole ring found in other porphyrin compounds for e.g. chlorophyll (with Mg) and heme (with Fe). B12 is produced by a limited number of microorganisms and found only in animal products. From food or other supplements, it pursues an intricate pathway of absorption and moves into the cells, where it assumes its central metabolic function as an important coenzyme for two enzymes in humans namely methylmalonyl-CoA and methionine synthase. Moreover, B12 could be one of the most important modulating factors impacting on gut functions and gut microbiota.

Industrial application

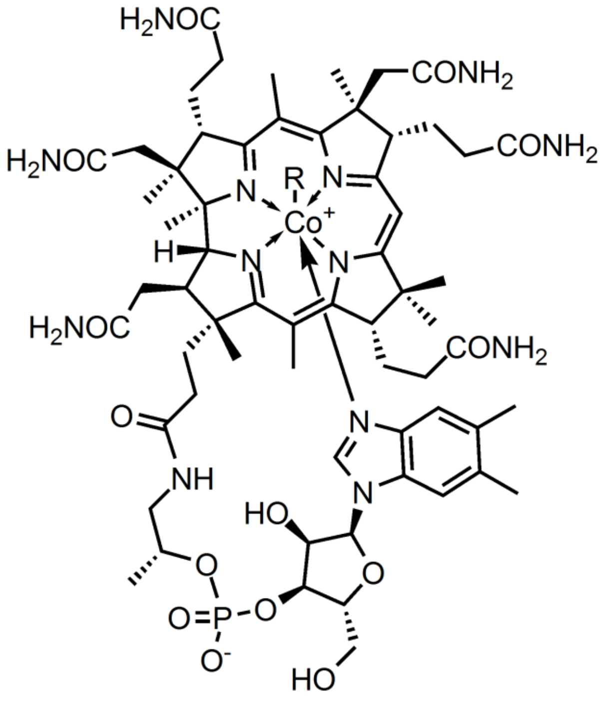
Vitamin B12 is a light red colour powder. It is used as a coloring agent in hams, sausages, ice creams, fish, and meat products.

1. It is used as nutritional supplement in food and beverage.
2. It is used as a nutritional therapy in treatment of pernicious anemia and vitamin B12 deficiency.
3. It is used as supplement in animal feed, agriculture, and poultry products such as sheep, cattle.
4. It is effective in treating hepatic necrosis, fatty liver and treat anemia.
5. It is available in various forms like nasal spray, injections and chewable tablets.

6. It is used for controlling homocysteine levels and treating cyanide poisoining.
7. CNB_{12} is the commonly produced B_{12} form and is used in the food sectors.

STRUCTURE OF VITAMIN B12

Vitamin B_{12} (cyanocobalamin) is a water soluble vitamin with complex structure. The empirical formula of cyanocobalamin is $C_{63}H_{90}N_{14}O_{14}PCO$. The structure of vitamin B_{12} consists of a corrin ring with a central cobalt atom. The corrin ring is almost similar to the tetrapyrrole ring structure found in other porphyrin compounds e.g. heme (with Fe) and chlorophyll (with Mg). The corrin ring has four pyrrole units. Cobalt present at the centre of the corrin ring is bonded to the four pyrrole nitrogen's. Cobalt also binds to dimethylbenzimidazole and amino isopropanol. Thus, cobalt atom present in vitamin B_{12} is in a coordination state of six (Fig.8).



R = 5'-deoxyadenosyl, Me, OH, CN

Figure 8: Structure of Vitamin B12.

BIOSYNTHESIS PATHWAY

Vitamin B12 is exclusively synthesized in nature by microorganisms. The pathway starts with succinyl CoA and used glycine as a precursors with the end product of cobalamin as vitamin B_{12} (5'-Deoxyadenosyl cobalamin). An outline of the pathway is demonstrated in Fig.9. The biosynthesis of vitamin B_{12} is compared with that of chlorophyll and hemoglobin.

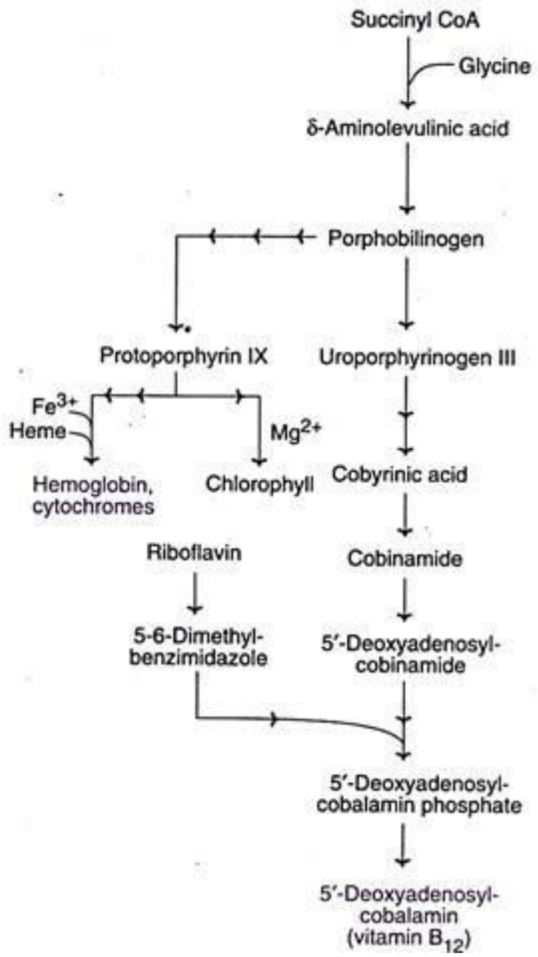


Figure 9: Biosynthetic pathway of Cyanocobalamin [Source: Adapted from Google images].

FERMENTATION PROCESS

Vitamin B_{12} is produced commercially by fermentation process. It was first obtained as a Streptomyces fermentation byproduct in the production of antibiotics (neomycin, streptomycin, and chloramphenicol). High-yielding strains were developed for mass

production. And at present, vitamin B12 is entirely produced through a fermentation process.

Organisms involved: Several microorganisms can be administered for the production of vitamin B12, with varying yields. Glucose is the most commonly used carbon sources for the production. Few examples of microbes and their corresponding yields are provided in the Table 1. The most commonly used microorganisms are — *Pseudomonas denitrificans, Propionibacterium freudenreichii, Streptomyces olivaceus,* and *Bacillus megaterium.*

Industrial fermentation: Production at industrial level has been performed using *P. freudenreichii* (anaerobic pathway) and *P. denitrificans* (aerobic pathway) with maximum yields of approximately 200 mg/l. Both species, but especially *P. denitrificans*, are attractive for biotechnological production because of their continuous growth with simple nutrient demand, their highest production, genetic accessibility and volumetric productivity.

Table 1. Microorganism and corresponding yields of vitamin B_{12}

Microorganism	Yield (mg/l)
Streptomyces olivaceus	3.31
Micromonospora sp	11.5
Bacillus megaterium	0.51
Propionibacterium freudenreichii	19.0
Pseudomonas denitrificans	60.0
Propionibacterium shermanii	35.0

Production of Vitamin B12 by using *Propionibacterium freudenreichii*

There are two different phases the microorganism involve into the production of vitamin B12, they are;

Anaerobic phase: This is a preliminary phase that may take around 2-4 days. In this phase 5′-deoxyadenosylcobinamide is generally produced.

Aerobic phase: In this phase, 5, 6-dimethyl- Benz imidazole is formed from riboflavin which gets integrated to form coenzyme of vitamin 5′-deoxyadenosylcobalamin.

Nowadays, some fermentation technologists have successfully clubbed both an aerobic and anaerobic phase to carry out the process continuously in two reaction tanks.

The bulk quantity of vitamin B_{12} production is mostly done by submerged type of bacterial fermentation with beet molasses medium content with cobalt chloride.

STAGES OF VITAMIN B_{12} PRODUCTION

P. denitrificans uses the aerobic pathway for vitamin B_{12} synthesis. Fermentation process is conducted with agitation and aeration, in the presence of nutrients, such as sucrose, yeast extract, and several mineral salts in the growth medium, at a temperature of 30^0C and pH of about 7 for a period of 6-7 days, providing a yield >150 mg/l.

The detailed fermentation process is given in Fig. 10. The fermentation is completely aerated during the exponential growth phase, the B12 production is increased by regulating the dissolved oxygen concentration (DOC) and also the level of CO_2 in the inlet gas. The process of fermentation initiated with a high DOC range (8–10%) to accept biomass production continued by a progressive reduction of DOC to 2–5% at 49–106 hours and below 2% at 107–168 hours. The aeration of the culture with a defined mixture of CO_2 and air, which enhanced the B12 yield by around 10% compared with the control. At the beginning the culture medium has to be supplemented with 40–200 mg/l of cobaltous nitrate and 10–25mg/l of DMBI. The addition of betaine or choline as methyl donor activates production of B12 precursor and results in enriched B12 production. Sugar beet molasses are often used as carbon source because of its high glutamate and betaine content.

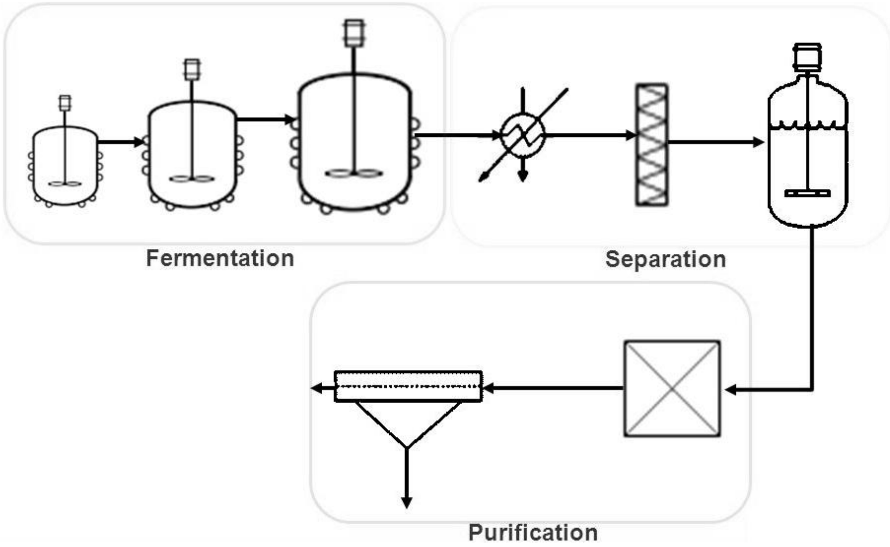


Figure 10: Vitamin B12 production flow diagram.

RECOVERY AND PURIFICATION

Downstream processing plays an important role in various stages of processing, namely purification, separation and packaging of the product. Fermentations produce a mixture of $AdoB_{12}$, OHB_{12} and MeB_{12} which are not separately isolated. OHB_{12} obtained via cobalamine are converted into chloro, sulfate or nitrate during the extraction stage. Whereas, $AdoB_{12}$, and MeB_{12} are isolated from the fermentation broth.

The cobalamine produced during the fermentation process are mostly bound to the cells. They can be completely solubilized by heating at 80-120^0 C for 30 minutes at pH 6.5 to 8.5. The presence of mycelium and solids are centrifiuged or filtered and the fermentation broth collected. The presence of cobalamins can be converted into stable cyanocobalamins. This product (cyanocobalmin) is pure around 80% and can be directly used for feed additive. It should be further purified around (95-98%) for medicinal use.

13.4.2 RIBOFLAVIN

Riboflavin (RF) [7,8-dimethyl-10-(1’-D-ribityl)isoalloxazine] commonly called as Vitamin B_2, lactoflavin or Vitamin G (Fig.11). It is a water soluble vitamin and considered as one of the most important micronutrient required for all prokaryotic and eukaryotic cells. As the part of Vitamin B complex, RF was the second most important vitamin ever identified. It serves as a main precursor of the flavin co-enzymes namely flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which are extremely important in many of the body's enzymatic functions for the transferring of electrons in oxidation-reduction reactions. The most important function of RF includes bringing energy from carbohydrates, proteins and fats that make up the diet. It is also necessary for the activation of other vitamins, including folic acid and pyridoxine. Nowadays, the commercial riboflavin products are produced biotechnologically through microbial biosynthesis, based on the use of *Ashbya gosypii, Bacillus subtilis,* or the yeast *Candida famata.*

NOTES

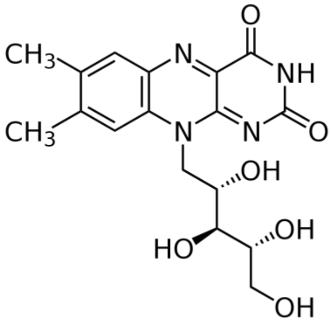


Figure-11: Riboflavin

As a chemical compound, riboflavin is a yellow-orange solid substance with poor solubility in water compared to other B vitamins. Visually, it imparts color to vitamin supplements.

INDUSTRIAL APPLICATION

Riboflavin and riboflavin phosphate are both stable to atmospheric oxygen and heat, especially in an acid medium. Riboflavin is regarded as being one of the more stable and strong vitamins than other types of vitamins. It is degraded by reducing agents and unstable with increasing level of pH value. While riboflavin is strong to the heat processing of milk, one of the main reasons of unstable in milk products and milk is from exposure to light source. Liquid milk exposed to light can lose around 20 and 80% of riboflavin content in two hours. It is used as yellow colouring for beverages and dairy food supplement. The industry which produces butanol, acetone and alcohol from molasses and grains has long been confronted with the problem of disposing fermentation residues. This residues are found to be great sources of riboflavin and other vitamins.

The fortifications of riboflavin rich foods are sometimes mandatory when the foods are commonly taken in various parts of the world. Riboflavin has been traditionally produced for feed and food fortification by chemical means but in recent years the biotechnological processes employing various bacteria, fungi and yeast have been found to be replacing chemical synthesis and commercially competitive.

METABOLIC PATHWAY OF RIBOFLAVIN BIOSYNTHESIS

The biosynthetic pathway of vitamin B_2 (RF) is demonstrated for the microorganisms *Eremothecium ashbyii* and *Ashbya gossypii* is shown in the Figure 12. The riboflavin overproduction in these organisms takes place mainly due to the integral nature of the synthesizing enzymes. Inhibition of vitamin B_{12} production by iron

content in yeasts and Clostridia which has no effect during the production of riboflavin in *A. Gossypii* and *E. Ashbyii.*

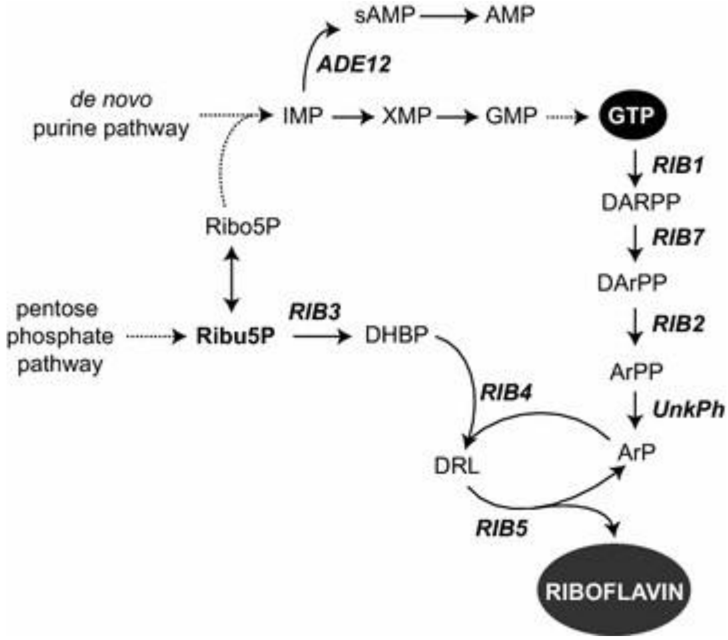


Fig 12: Biosynthetic pathway of riboflavin [Source: Amaro *et al.,* 2015)

FERMENTATION OF THE RIBOFLAVIN

Organism used: Three groups of microorganisms have been found to synthesize riboflavin in significant amounts: *Lactococcus lactis, Corynebacterium ammoniagenes* and *Bacillus subtilis* helps in characterising the riboflavin biosynthetic pathways. *Pichia guillermondii* (used as yeast model), *Debaryomyces hansenii, Candida famata, Candida albicans, Schwanniomyces occidentalis* and *Ashbya gossypii* are the natural over producers for riboflavin synthesis.

Commercial fermentation processes for riboflavin production are relatively recent, having been developed since about 1940. The Ascomycete processes by using *R ashbyii* and *A. gossypii* are now assuming the most important position in the riboflavin manufacturing field.

Media: The riboflavin fermentation process by *Ashbya gossypii*, the media comprising of glucose (corn sugar), animal stick liquor (by product of wet rendering), corn steep liquor (by product of wet corn milling) prepare in a mixing tank. The composition of the ideal medium for riboflavin fermentation is as follows (g/liter): glucose, 20.0;

peptone, 5.0; yeast extract, 5.0; malt extract, 5.0; $MgSO_4.7H_20$, 0.2; K_2HPO_4, 0.2.

Steps involved in fermentation: The medium is forced at a controlled rate through high pressure steam jet heater, the solution is almost heated to 135^0 C. The hot solution disseminate through insulated pipes to maintain the high temperature for 5 minutes, then through the supplementary coils or pipes surrounded by cold water to cut down the temperature to 82° to 86° F. Through sterilized steam pipe, the cooled solution is moved to a sterile fermentation vessel. This is one of the closed tank equipped with a coil or jacket by which the tank may be maintained at a steady temperature of 28°C. The bottom of the tank contains fine-perforated coils or porosity stone through which sterile air is supplied. A mechanical agitator aids in providing sufficient air distribution. A small volume of a day-old culture of *Ashhya gossypii* is added to the sterile culture medium in the tank, and sterile air is passed through the air distribution system at a volume of one-fourth to one-half per volume of medium per minute. At the tim of fermentation media pH drops from 6.5 to 4.5. At this point the microorganisms grow rapidly, but no significant amount of riboflavin is synthesized. Then both riboflavin production and pH increase and riboflavin formation terminates at approximately pH 9.5. At fourth day, the maximum yield of riboflavin has been obtained, and the culture medium has acquired as a beautiful, intense yellow color. Glycerol, sunflower oil, whey and several combinations of these substrates can also be used for riboflavin production.

A potent riboflavin concentrate, potentially suited to enriching livestock and poultry feeds, can be obtained by evaporating the water from the fermented medium to form a sirup of about 30 percent solids. Later, the sirup is converted to a dry powder by such conventional equipment as a spray or drum drier. The drum drier contains a pair of cylindrical rolls, horizontally mounted, which are steam heated. The sirup is regularly fed to the valley between the rolls, and the rolls circulate in opposite directions and a thin film of sirup adheres to each other. The water evaporates rapidly before a revolution is completed, and the dry materials are scraped off by knives, is transferred to bagging equipment. Whereas, in spray-drier, the sirup is sprayed into a chamber over hot air is passed; the air largely absorbs the water; and the dried part riboflavin concentrate is mechanically removed to packaging

equipment. Pure crystalline riboflavin may be recovered from the fermented solution.

Check your Progress -2
Note: Write your answer in the space given below
c. List out the microbes used for vitamin B12 production.
d. Discus the fermentation parameters of riboflavin production..
………………………………………………………………………………

13.5 LET US SUM UP

In this chapter, you have been leant about the antibiotics and their uses. Microbial production of penicillin and streptomycin antibiotics. The basid raw materials and the fermentation parameters involved in the fermentation process also known through this chapter. How microbes used for vitamin production, especially vitamin B12 and riboflavin? the metabolic pathways involved in the vitamin biosynthesis and the important media, fermentation parameters for vitamin production also studied through this chapter..

13.6 UNIT - END EXERCISES

1. Explain the method of Streptomycin production.
2. Discuss about industrial production of vitamin B12.

13. 7ANSWERS TO CHECK YOUR PROGRESS

a) Penicillin is an antibiotics inhibits the cell wall peptidoglycan cross linker formation gram negative bacteria.
b) Streptomycin is produced in aerobic fermentation of *S. griseus*
c) Streptomycin sp.
d)

13.8 SUGGESTED READINGS

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UNIT -XIV: FERMENTATION ECONOMICS

Structure

14.1 INTRODUCTION

In this chapter deals with the concept of fermentation economics, cost of the product, market value and potentials, factors influencing the market potentials of the product including media, incubation time, sterilization methods, unit operation, labor cast, containment facilities, and the total investment are to be discussed. In addition, the maintenance of legislations on production of antibiotics and recombinant proteins are also to be discussed.

14.2 OBJECTIVES

After going through the unit you will be able to;

- Understand the values of fermentation economics.
- Know factors influencing the product cost.
- Understand the legislative recommendations for the production of antibiotics and recombinant proteins.

14.3 FERMENTATION ECONOMICS

Fermentation is one of the oldest known chemical processes. Fermented foods and alcoholic beverages have been produced for thousands of years, but it is only in the last four to six decades that biotechnology and chemical engineering have been applied on a large

scale production of fermentation products such as antibiotics, amino and organic acids, flavoring agents, solvents, and vitamins.

The economics of fermentation is depends the industrial production of metabolites with its cost of production, if the product cost is too high in direct chemical synthesis, microbial fermentation is cheapest one. There are many reasons for the commercial microbial fermentation is cheaper than adverse chemical synthesis, may be due to relatively high efficiency, coupled with availability and cost of agricultural raw materials, allows use of biological systems to produce needed chemical products.

According to a new report published by Allied Market Research, titled, Fermentation Products Market by Type, Feedstock, Process, and End-user Industry: Global Opportunity Analysis and Industry Forecast, 2017-2023, the global fermentation products market was valued at $149,469 million in 2016, and is projected to reach at $205,465 million by 2023, growing at a CAGR of 4.7% from 2017 to 2023. The alcohols segment dominated the market in 2016, accounting for more than half of the market share, in terms of volume. Similarly, production of enzymes themselves has become an important industrial process. Large-scale conversion of glucose to high-fructose corn syrup requires equally large-scale production of the needed enzyme—glucose isomerase.

Prior to commercialization of any products, extensive economic validation is necessary. it includes,

1. Unique characteristics of fermentation processes
2. Use of agricultural raw materials,
3. High-energy use per unit of product,
4. Need for aseptic operation, and
5. Require involvement of biologist, chemical engineers, and other engineering disciplines needed for plant construction and startup.

It is assumed that, if a totally new facility is to be built, site selection is a basic requirement, following with process design. Process design follows both conventional engineering needs as well as those derived from unique biochemical inputs from laboratory and pilot plant data. Further, instrumentation and mechanical designs, certain unique control and cleanliness requirements are other needs. It is best to consider immediately from the outset that the plant will most likely fall under jurisdiction of one or another governmental regulatory body

since a food or pharmaceutical product may be manufactured. A clean environmental regulation is important for successful operation of the plant. Costly correction or modification may then be necessary.

The capital and gross operating cost of a "conventional" fermentation plant is roughly independent of the product produced. At current prices, as a first approximation for a grass-roots fermentation and finishing plant cost, one can use a figure per liter of installed fermentation capacity. The value within this range depends upon scale and complexity. The economics of scale-up of capital costs will likely have an exponent of 0.75 for the fermentation section of the plant relative to the 0.6 exponent commonly used for a first approximation in processing plants.

The fermentation operating costs per unit volume per unit time will vary somewhat but will hold generally within a reasonably narrow range. That is based on,

1. Use of an exotic raw material,
2. The cost of labor,
3. Utilities, and materials would not vary greatly,
4. Processing or extraction cost range,
5. The capital and operating costs of all auxiliary operations for fermentation and extraction
6. Total installation cost and cost of operation.

Thus, the unit cost of the bulk product is much a function of fermentation yield and fermentation cycle time.

14.4 MARKET POTENTIAL

The objective of any successful fermentation process is the ability to produce a fermentation product. Thus the product must be sold to recover all the costs along with desired profit. But manufacturing should be done in accordance with the market demand.

A process is considered commercially successful when it produce high yield of any fermentation product. The selling price of the product should be such that it recovers the production cost with satisfactory profit. On the other hand, the demand or market potential of the product decide to what extent product should be produce. There are two ways to equate demand and supply ration.

1. First, whether the market for the product may already exist or not and the same product has previously been sold by others or presently under selling.
2. Second, what is the potential of establishment of market for a newly discovered fermentation product which has not been sold previously in the market i.e. it is required to establish a market for that product

Assignment of any new fermentation product in the market is a time consuming and costly affair as it require approval by the government agencies like Food and Drug Administration or other before they introduced in the market. Sometimes, due to fewer uses and less demand of certain fermentation products, it not easy to place it in the market. However, the market of some products already exists due to consumers' acceptability and long term persistence of these products in the market. So that, market potential, production cost, market demand and sale and competitions amongst existing products are important aspects to be consider at the time of launching a new product in the market.

Many fermentation products are not sale directly in the market but used internally for an industrial concern. For example, a company that manufactures and sells product A might produce B by fermentation of C, followed by a chemical conversion of the B to A. On the other hand, a fermentation product can be sold directly to a different industrial used which chemically transforms the fermentation product before sale in the market. If so, it is possible to reduce the extra costs of advertising, packaging, and distribution to retail outlets which apply to fermentation products marketed directly to public. Addition to this, generally it does not require any trademark, proprietary name and generic name. Foreign sales present an added market potential for many fermentation products. For example, exporting product and selling in the foreign marker and on the other hand, manufacturing the same product in foreign country and selling in their market makes a wide difference in the profit.

14.5 FERMENTATION AND PRODUCT RECOVERY COSTS DIRECTLY RELATED TO THE FOLLOWING PARAMETERS

1) Medium constitute: Usually, the costs of different constituents of the production medium decides the competitive position and potential profits

of a fermentation product. For example, inoculum media are usually less expensive because they are designed to promote fast growth of the producer instead of converting a large amount of carbon substrate in the product. At the same time they are require in less volume. In contrast, production media which are aimed to produce the product required in more volume and may time high-cost of a single medium can affect the selling price the fermentation product. So, to reduce the medium cost the alternative low-cost replacement of certain medium constituents while formulating the production media. As a matter of fact every components are directly or indirectly influence the economics of the fermentation process. For example, because of the world political situation, if the availability and cost of cane black-strap molasses becomes too high, one cannot afford to use this as a carbon source. Under such situations there should be provision for an alternative carbon source which is cost effective and made available easily. One should remember that sometime he use of an alternate medium requires the use of a different fermentation microorganism or strain of the organism.

Several media need decontamination to remove some contaminated chemical species as they influence the product accumulation and many times make the recovery process difficult. For example, certain metal ions can be removed by ion-exchange resins. In addition, many media requires pretreatment before they employed. For example, starch must be pre-treated with amylase to release fermentable sugars and proteins must be degraded by proteolytic enzymes to release amino acid for yeasts growth. Acid or alkali are required to adjust the pH of the medium during production process. As these reagents are not so costly, but considerable reagent may be required for the adjusting the pH value of a large volume of medium which many time lead to a considerable high cost. Similarly, media rich in protein components produce foam and increase the chances of contamination. Under such condition controlling of foam either by chemical antifoam agents of with mechanical device also add the cost of production. Some time the raw material used for media formulation will affect the product recovery process or affect the product purity. So, the quality and the impurity other fermentative ability of the raw material also consider for media formulations.

2. **Fermentation incubation period:** Fermentation process with short incubation period are less costly compared to processes with prolonged incubation with reference to the inoculum build up and to production. One can harvest more batch in case of short incubation period fermentation with a larger turnover of fermentation. If a fermentation requiring long

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incubation periods require,1) additional requirement, 2) extra labor cost and 3) have more possibility of getting contamination .

3. **Contamination and sterilization:** Many fermentation processes are more chance to contamination compared to protected fermentation processes. For example, foaming is the common problem, prolonged incubation, the poor competence of product producing organisms with contaminants for fermentation nutrient, lass of metabolites due to contaminants, and bacteriolytic phage infections.

Those fermentation processes in which media sterilization cost is not affordable certain alternative methods to control the growth of contaminants are adapted. These are a) adjusting low pH of the medium, b) selecting a substrate poorly amenable to attack by contaminants c) partial sterilization of the medium, and d) selecting certain chemicals which retard the growth of contaminants

If the fermentation medium is severely contaminated, then it must be discarded and this adds more cost to the production. Many time mutation in genetically unstable production strain results in a population of low-yielding cells

4. **Yields and product Recovery:** High yield and adequate recovery of any fermentation product is of prime importance in any fermentation process. For any fermentation product, high yield with proper recovery and purity affect its position on the open market. For any fermentation product, to maintain a competitive market position it requires to have continuous research and product development program to improve and increase its yields and a better methods of product recovery. The cost of these important downstream processes very high but extremely important too.

5. **Product purity:** The purity of fermentation product decides its future stand and long term market value. The costs of product is directly associated to its purity. For example, some antibiotic preparations useful for human applications must be sterile and free from pyrogens. On the other hand, some products which are used with other antibiotic preparations, are sold in crude form for mixture with animal feeds. Purification steps like solvent extraction and followed by crystallizations for a fermentation product significantly contribute to the overall costs of the product.

6. **Waste disposal:** The overall production cost of any fermentation product is influenced by outflow of capital used for waste treatment and disposal and it depends on following two aspects; a) acceptance of fermentation waste with or without pretreatment by municipal waste-treatment facilities or any other local service providers or , b) maintenance of waste water treatment plant by the fermentation

As per the environmental regulations, it is not possible to dispose fermentation wastes into rivers, streams or any other stagnant water bodies for long time. In certain case, for example, waste generated in the fermentation processes which exploit plant or animal pathogens as the fermentation organisms requires an additional expense of sterilization before discarding to avoid biohazards. There are many sources through which waste is generated. There are; a) waste generated from the actual fermentation ; b) wastes from recovery processes; c) waste form cleaning water ; d) waste from cooling waters, and; e) waste from various staged of product recovery and purification etc.

Many times it is feasible to use waste water for some other purposes, for example, cooling waters can be utilized in media makeup for similar or other fermentations. It is possible to recover by-products from the fermentation wastes. For example, riboflavin from the acetone-butanol fermentation, which can reduces the product cost.

7. **Labor cost:** The cost which incur to pay for non-technically and technically trained personal working in any industry at all level of is called labor costs. This include labor related to,

a) *Cultures handling*
b) *Inoculum*
c) *Production*
d) *Product recovery and purification*
e) *Packaging of the product*
f) *Steam production*
g) *Equipment maintenance and cleaning*
h) *Administration etc.*

This labor costs depends on type of fermentation process (batch of continuous) level of containment to be employed, type of organisms under use (genetically engineered or non-genetically engineered), volume of the product etc.

8. Research costs: To maintain a competitive commercial stand, the cost picture of a fermentation process which includes expenses incurred in the

research and discoveries for the development of the process should be clear. The high cost research without positive outcome in terms of innovations and novelty is of no use for industries facing financial crisis. Investing in research is a long term venture. It give incredible financial success provided it is done in a right direction.

9. Capital Expenditure: Commercial production of a newly developed fermentation is expensive and may require outlay of capital investment. Establishment of expensive fermentation equipment which are in continuous use for the production along need the maximum requirement for capital expenditure with other facilities. Thus, a new fermentation set up requires the prompt of existing facilities along with the construction of additional facilities. A similar condition may take place for product recovery and purification facilities. A newly developed fermentation process may have need of the setting up of newly designed fermentation equipment. Installation of this equipment to a great extent adds additional cost to the fermentation costs

10. Patent position: The profit picture of any fermentation process or product is remarkably influenced by sound patent position. Patent position of process or product helps in reducing the extensive commercial competition which remarkably influences the profit picture. A promising patent position provides greater potential for cost recovery and an adequate profit. The costs for getting a patent are relatively small. However, in case any infringement proceedings are instigated, the patents become a costly. Patents, can yield revenue to the holder through its utilization in the production and marketing of the fermentation product. For a return of royalties, it is possible to licensed then patent to other fermentation companies and even competing companies. Even after doing so, the patent holder can still produce the fermentation product. Mostly, the patent holder may not have the capital or facilities to produce and market the product. In such cases, it may be to the advantage of the patent holder to license the patent without producing the fermentation product, or to sell the patent outright.

Check your Progress-1

Note: Write your answer in the space given below

a. Define market value.

b. Compare media cost vs product cost.

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14.6 PROCESS ASSESSMENT

The prior deliberations must be evaluated keeping in mind the present and future market conditions to assess the economic potential for any fermentation process development. It is equally important to re-evaluate the process during commercial production. Following aspects should be considered during overall evaluation of any fermentation process.

a) estimation of present and future availability and price of fermentation substrates
b) costs of labor
c) overhead expense
d) public demand for the product
e) competition in the market
f) potential for bettering yields
g) product recoveries
h) capability and facilities to meet market demands for the product
i) consideration of all present and future costs
j) selling price and desired profit for the product

All of these extent must be studied carefully to decide before the production of the fermentation products.

14.7 BIOTECHNOLOGY INDUSTRIES IN INDIA

The Indian Biotechnology sector is one of the major knowledge based sectors in India and is contributing significantly to shaping India's rapidly growing economy.

Biotechnology is a recent development in the field of science and technology. Biotechnology as the name suggests is the combination of biology and technology and is an applied science. It is concerned with variety of subjects including biochemistry, genetics, microbiology, chemistry and engineering. Biotechnology has wide areas of application in agriculture, animal husbandry, development of medicines and vaccine, treatment of prolonged disease, pollution control and waste management and ecological conservation, energy production and conservation and many more.

The Indian biotechnology sector is one of the fastest growing knowledge-based sectors in India and is expected to play a key role in shaping India's rapidly developing economy. With numerous

comparative advantages in terms of research and development (R&D) facilities, knowledge, skills, and cost effectiveness, the biotechnology industry in India has immense potential to emerge as a global key player.

The sector can be divided into the segments of bio-pharmaceuticals, bio-services, bio-agriculture, bio-industrial and bio-informatics. Nearly 64 per cent of the biotech companies operate in the bio-pharma sector, followed by the bio-services (18 per cent), bio-agri (14 per cent), bio-industrial (3 per cent) and lastly the bio-informatics sector (1 per cent).

Market Size

Biotechnology in India is based on a network of nearly three hundred national laboratories and about an equal number of universities. The national laboratories operate under various departments or agencies of the Government of India, predominantly the Council of Scientific and Industrial Research (CSIR), the Indian Council of Agricultural Research (ICAR), the Indian Council of Medical Research (ICMR), the Department of Science and Technology and the Department of Biotechnology, among others. All of them have contributed to the emergence of biotechnology as major technological area in the country.

The biotechnology sector in India is expected to achieve revenue of US$ 11.6 billion by 2017, growing at a compound annual growth rate (CAGR) of 22 per cent, according to a recent report by Ernst & Young (E&Y). The key growth drivers of the US$ 4.3 billion industry include strong domestic demand for biotech products, growth in contract services, focus on R&D initiatives and strong government support for the sector.

Revenue from biotech exports reached US$ 2.2 billion in FY13, accounting for more than half (51 per cent) of total industry revenues. During FY05 and FY13, revenue from exports increased at a CAGR of 25.1 per cent to US$ 2.2 billion from US$ 0.4 billion.

Investments

Investments, together with outsourcing techniques and exports, are crucial for growth in the biotech sector. According to data released by the Department of Industrial Policy and Promotion (DIPP), the drugs and pharmaceuticals sector has attracted foreign direct investment (FDI) worth Rs 54,780.65 crore (US$ 8.81 billion) between

April 2000 and September 2013. Some of the major investments in the sector are as follows:

- Biocon plans to set up an institute in Bengaluru to train graduates in skills required for finding employment in the fast-growing biotechnology industry. The institute, starting in January 2014, will offer a 16 week certificate-programme in partnership with California-based Keck Graduate Institute.
- Bharat Biotech launched the world's first clinically proven conjugate Typhoid vaccine 'Typbar-TCV', which offers long-term protection and can be given to children as young as six months.
- Roselabs Biosciences Ltd has set up fully integrated pre-filled syringes (PFS) manufacturing unit in Ahmedabad with an investment of Rs 400 crore (US$ 64.40 million).
- LifeCell International Pvt Ltd has won funding of Rs 35 crore (US$ 5.63 million) from Helion Venture Partners. The investment will be used to increase LifeCell's market awareness and penetration in the country, said Mr Mayur Abhaya, CEO and MD, LifeCell.
- HLL Biotech Ltd (HBL), a subsidiary of HLL Lifecare Ltd, has entered into a long-term supply and technology license agreement with the Institute of Immunology, Zagreb (IMZ), Croatia, for the manufacture of measles vaccine in India. Under the partnership agreement, IMZ will transfer the technology to manufacture bulk measles vaccine to HBL.

Government Initiatives

The Government of India has set up the Department of Biotechnology under the Ministry of Science and Technology in 1986. Public funding in the science and technology sector has been on the rise by 8 times in the last 20 years. The Indian government also provides several fiscal initiatives including relaxed price control for drugs, removal of foreign ownership limits, subsidies on capital expenses, and tax holidays for R&D spending.

The Indian Government's increased focus on the country's biotechnology industry has enabled it to grow at a CAGR of approximately 20 per cent over the past decade.

Besides the central government initiatives, individual states are also doing their bit to promote the biotechnology industry. Karnataka takes the lead and the state's revised biotech policy offers many fiscal incentives and concession to prospective investors in the industry.

Some of initiatives taken by the Government to further promote the sector are as under:

- The Ministry of Science and Technology, Government of India and RIKEN, Japan's largest research organisation have signed memorandums of understanding (MoU) to launch joint research programs in the fields of biology, life sciences and material sciences.
- UK Trade and Investment (UKTI) and the Association of Biotech Led Enterprises (ABLE) have signed a MoU to encourage and develop collaborative opportunities between Indian life sciences organisations and the UK.
- The Drugs Controller General of India has approved Biocon Ltd to market its biosimilar 'Trastuzumab' developed jointly with the US drug-maker Mylan, for treating breast cancer. "This is a major milestone for both partners as it is the world's first biosimilar trastuzumab to be accorded regulatory approval," said Ms Kiran Mazumdar Shaw, Chairperson and Managing Director, Biocon.
- The Government of India plans to set up National Institute of Biotic Stress Management for addressing plant protection issues will be established at Raipur, Chhattisgarh.

14.7 EFFECTS OF MAINTENANCE LEGISLATION ON THE PRODUCTION OF ANTIBIOTICS AND RECOMBINANT PROTEINS

Development and regulatory approval of any product to be added to food or used as medicine is a multifaceted, extensive and generally costly process. Regulatory approvals for food additives vary significantly from country to country. We discuss here regulatory approval pathways for food antibacterials in the United States (US) because this country represents by far the major potential market for these products and because its regulatory review process can be relatively simple and fast (relatively inexpensive), compared to the regulations in most other countries. In the US, any substance to be intentionally added to food is a food additive and must be subjected to premarket review and approval by the FDA under the Federal Food, Drug and Cosmetic Act (FFDCA; the “Act”), unless the substance meets a listed exemption in the Act, or is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use. GRAS’ (Generally Recognized As Safe) is an FDA designation that a chemical or substance added to food is considered safe by experts, and so is exempted from the conventional

premarket approval process by FDA. The developer of the new substance (the Notifier) conducts an analysis of safety and utility of its product using scientific procedures including corroboration from publically available information, and determines and documents that the substance is GRAS as specified by the FDA's Final Rule for GRAS Notices.

The Notifier voluntarily submits its GRAS conclusion to FDA for review and comment. The FDA can either reject Notifier's conclusion of safety, cease to evaluate the submission upon request by the Notifier, or, ideally, issue a "No Questions" letter to Notifier. The latter verifies that the FDA agrees with Notifier on its conclusion that the substance is GRAS and equates to marketing allowance by FDA for the substance. The FDA may conduct the GRAS review on its own for certain types of food treatments, or solicit input from the United States Department of Agriculture (USDA) if the substance is to be applied to USDA-regulated products such as meat and egg products. The GRAS pathway can be used for substances added to human food or animal food, as well as for animal feed ingredients. In addition to the FDA, an alternative body capable of conducting GRAS reviews is the Flavor and Extract Manufacturers Association (FEMA), which is the national association of the United States flavor industry. FEMA works with legislators and regulators to assure that the needs of members and consumers are addressed and can provide GRAS guidance, although their function is restricted to flavor substances.

The GRAS designations fall into several categories, the most relevant of which for food antibacterials is "Food Processing Aid." Substances added to food are classified as Food Processing Aids if they provide a rapid yet temporary effect, degrade and become part of the food matrix and thus have no functional effect on the food. If FDA accepts such a designation based on the evidence provided, the designation allows the manufacturer to avoid listing the substance on the treated food's product label; thus, there is no labeling requirement for the substance. Food additives or food ingredients, on the other hand, are typically persistent, are essential to or can modify the food's functionality, and need to be listed on the product label. Facilitated regulatory pathways similar to GRAS exist also in a few other countries, for example Canada, Mexico, Australia, and New Zealand. In yet other territories, including countries of the European Union and Japan, approval of a new food additive involves a process similar to the USA's pre-market

review of a new non-GRAS substance, requiring extensive toxicity/safety studies. Nomad Bioscience is the first company to successfully obtain FDA concurrence for GRAS designation of its plant-made bacteriocins, such as colicins, as food antimicrobials. In its first GRAS notice (GRN 593), the following arguments were used to support safety and suitability of colicins made in food species hosts as food antimicrobials. In 2015, based on Nomad's submission, FDA accepted colicins as first-in-class GRAS antimicrobials for controlling pathogenic *E. coli* in fruits and vegetables (GRN 593), and in 2017, FDA and USDA accepted colicins as antimicrobials for controlling *E. coli* in meat products (GRN 676). In both cases, the Agencies agreed with the "Food Processing Aid" definition and USDA has added colicins to its FSIS Directive 7120, which is a list of safe and suitable ingredients allowed for use in the production of meat, poultry and egg products (FSIS Directive 7120.1, Revision 42). Subsequently, Nomad also filed GRAS notices for *Salmonella salmocins* (GRN number pending) and *C. perfringens* bacteriophage endolysins (GRN 802). Independently, Nomad also submitted a GRAS notice for the use of *N. benthamiana* (non-edible) production host for the manufacture of colicins (GRN 775), which led to a "No Questions" letter from FDA.

Regulatory experience to date suggests that additional plant-made colicins, phage-derived endolysins, other bacteriocins, defensins, etc., could also gain rapid marketing allowance. In particular, in 2018 Nomad received 'No Questions' letter from FDA for a product candidate in another category of food treatments, the natural sweeteners/taste modifiers thaumatins (GRN 738). As long as the GRAS notice describes a natural or nature-identical substance, GRAS designation is a relatively simple, fast and inexpensive way for obtaining regulatory review and product marketing allowance, as evidenced by the success of Nomad's GRAS submissions to date. Based on this experience, we see a potential GRAS allowance 'space' for multiple classes of natural proteins such as:

- Colicins/bacteriocins-based feed treatments as antibiotic alternatives for controlling bacteria in animals during life or prior to harvesting;
- Antivirals such as antibodies or lectins (griffithsin) added to food (e.g., control of norovirus, rotavirus, influenza);
- simple – Functional (medicinal) foods to control bacteria or viruses in the gastrointestinal tract;
- Bacteriocins as topical/oral treatments;

- Natural proteins (e.g., thaumatin) as non-caloric sweeteners, taste modifiers, etc.

Natural products that are used to treat food but that have no functional effect on food (food processing aids) are obviously the easiest cases to take through the GRAS process. For colicins, the inherent safety of these proteins was supported in part by the fact that colicins and colicin-like bacteriocins are very sensitive to proteases, and any traces of these proteins remaining in the treated food would be rapidly degraded in the stomach and duodenum; Nomad provided FDA with extensive data on gastroduodenal degradation of colicins in its dossier. Future uses of bacteriocins as food treatments to control bacteria in the human or animal gastrointestinal tract would likely require additional data on bacteriocin safety, bioavailability, and their functionality in the intestinal lumen.

Table-1: Examples of therapeutic recombinant proteins obtained by heterologous expression in *E. coil*

Recombinants Proteins/Antibiotics	**Functions (Clinical use)**
Aldesleukin(interleukin-2)	Melanoma and renal cancer treatment
Anakinra(interleukin1(IL1)receptora ntagonist)	Rheumatoid arthritis treatment
Calcitonin (salmon calcitonin)	Post menopausal osteoporosis treatment
Denileukin diftitox (interleukin-2 an Diphtheria Toxin fusioned)	T-cell lymphoma treatment
Filgrastim (analog to the granulocyte colony-stimulating factor)	Neutropenia treatment (as consequence of AIDS, chemotherapy, bone-among others)
Filgrastim pegylated	Neutropenia treatment (as consequence of AIDS, chemotherapy, bone- marrow transplantation, among others)
Growth hormone(GH)	Prader-Willi and Turner syndromes
Glucagon	Hypoglycemia
Glucarpidase (bacterialcarboxypeptidaseG2)	Control of methotrexate conc. in patients with deficient renal function
Insulin (inhalation)	Diabetesmellitus treatment
Insulin (fast-acting)	Diabetes
Insulin (zinc extended)	Diabetes mellitus treatment
Interferon-α2a	Chronic hepatitis C. chronic myelogenous leukemia, hairy cell leukemia, Kaposi's sarcoma

NOTES

Interferon-α2b	Chronic hepatitis C. chronic myelogenous leukemia, hairy cell leukemia, Kaposi's sarcoma
Interferon- α2b pegylated	Chronic hepatitis C. chronic myelogenous leukemia, hairy cell leukemia, Kaposi's sarcoma
Interferon-β1b; Interferon-β1b	Multiplesclerosis Chronic granulomatous disease, severe osteopetrosis
Mecasermin (insulin-likegrowthfactor1)	GH and IGF1 deficiencies GHandIGF1deficiencies
Mecasermin rinfabate (insulin-like growth factor I and its binding proteinIGFBP-3)	Acute decompensated
Nesiritide (B-type natriuretic peptide)	Heart failure (ADHF) treatment
Oprelvekin (interleukin 11)	Prevention of severe thrombocytopenia (patients in chemotherapy)
OspA (Outer surface protein A fragment from *Borrelia burgdorferi*)	Lyme disease vaccine
Palifermin (truncade keratinocyte growth factor)	Treatment of oral mucositis in (patients undergoing chemotherapy)
Parathyroid hormone	Treatment of osteoporosis and hypoparathyroidism
Pegvisomant, modified GH (prevent GH binfing to receptor)	Acromegaly treatment
Ranibizumab (Mab fragment)	Age related macular degeneration
Reteplase (plasminogenactivator)	Acute myocardial infarction treatment
Somatropin, tasonermin	hGH deficiency treatment
Tasonermin (cytokine)	Soft sarcoma treatment
Urate oxidase, PEGylated	Gout
Teriparatide. Parathyroid hormone	Severe osteoporosis treatment
Glucagon like peptide 1, Liraglutide Insulin	Diabetes mellitus treatment
(PEGfilgrastim) Neulasta	Cancer related infections
Lucentis (Ranibizumab)	Macular degeneration
Erythropoietin	Promoting red blood cell formation in the treatment of anemia
Factor VIII	Helping blood clots form in hemophiliacs
Filgrastim and sargramostim (blood cell–stimulating bone marrow factors)	Boosting white blood cell counts after radiation therapy or transplantation

Insulin	Treating diabetes
Insulin-like growth factor 1 (IGF1)	Treating certain growth problems
Interferon (alpha)	Treating hepatitis B and C, genital warts, certain leukemias and other cancers
Interferon (beta)	Treating multiple sclerosis
Interleukin-2	Killing tumor cells
Somatotropin	Treating growth hormone deficiency
Tissue plasminogen activator (t-PA)	Dissolving blood clots to prevent heart attacks and lessen their severity
Erythromycin A, Tetracycline, Vancomycin, Streptomycin, Nisin A, Reuterin	Antibacterial
Amphotericin B, Ieodoglucomide C	Antifungal
Bleomycin	Squamous cell carcinomas, Hodgkin's lymphomas and testis tumors
Ddaunorubicin	Acute lymphoblastic or myeloblastic lymphoma
Rapamycin	Immunosuppressive, antifungal, antitumor, neuroprotective, neuroregenerative, and lifespan extension activities, growth inhibitory activity against several fungi
FK506	Immunosuppressive, antifungal, anti-inflammatory, neuroprotective and neuroregenerative activities, rheumatoid arthritis treatment
Cahuitamycins	Inhibitors of Acinetobacter baumannii biofilms
Transforming growth factor-b1	Heterologous protein delivery in foodstuff or in the digestive trac
Salinamides A and B	Efficient protein secretion system such as Sec pathway and twin-arginine-translocation (Tat) pathway

Check your Progress -2
Note: Write your answer in the space given below
a. List out the microbes used for vitamin B12 production.
b. Discus the fermentation parameters of riboflavin production..
………………………………………………………………………………

15.8 LET US SUM UP

Through this chapter, you have learned about the concept of fermentation economics, cost of the product, market value and potentials, factors influencing the market potentials of the product including media, incubation time, sterilization methods, unit operation, labor cast, containment facilities, and the total investment are to be studied. In addition, the maintenance of legislations on production of antibiotics and recombinant proteins are also studied.

15.9 UNIT - END EXERCISES

1. Explain economics of microbial fermentation.
2. Discuss about safety measures in recombinant proteins production.

15.10 ANSWERS TO CHECK YOUR PROGRESS

1. Bacillus sp., Streptomyce sp.
2. ~~2.~~
3. ~~3.~~

1.11 SUGGESTED READINGS

1. Ledesma-Amaro, R., Serrano-Amatriain, C., Jiménez, A. and Revuelta, J.L., 2015. Metabolic engineering of riboflavin production in Ashbya gossypii through pathway optimization. Microbial cell factories, 14(1), p.163.
2. Ottaway, P.B., 2008. The stability of vitamins in fortified foods and supplements. In Food Fortification and Supplementation (pp. 88-107). Woodhead Publishing.
3. Fang, H., Kang, J. and Zhang, D., 2017. Microbial production of vitamin B 12: a review and future perspectives. Microbial cell factories, 16(1), p.15.
4. Höllriegl, V., Lamm, L., Rowold, J., Hörig, J. and Renz, P., 1982. Biosynthesis of vitamin B 12. Archives of microbiology, 132(2), pp.155-158.

5. Najafpour, G., 2015. Biochemical engineering and biotechnology. Elsevier.
6. Ziemons, S., Koutsantas, K., Becker, K., Dahlmann, T. and Kück, U., 2017. Penicillin production in industrial strain Penicillium chrysogenum P2niaD18 is not dependent on the copy number of biosynthesis genes. BMC biotechnology, 17(1), p.16.

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MODEL QUESTION PAPER

BIOPROCESS TECHNOLOGY

Time : 3hrs **PART - A** Max marks:75

I. Answer all the questions not exceeding 50 words each.(10 x 2 = 20)

1) Define – Fermentation
2) Define auxotrophy
3) Write the agitator.
4) What do you know fed-batch culture?
5) Write any four roles of a fermenter
6) Name any two antifoam compounds.
7) List out the merits of internal examulti-stage fermentation.
8) Distinguish between aerobic and anaerobic fermentation.
9) Discuss the uses of ethanol?
10) Vitamin B12 producing microbes.

PART – B

II. Write any FIVE questions of the following not exceeding 650 words each.
(5 x 5 = 25)

11) Briefly describe the secondary metabolites of industrial important.
12) Write in detail about Air-lift fermenter
13) How do you differentiate solid state fermentation from submerged fermentation.
14) Explain the basic steps involved the purification of an enzyme.
15) Give an account on the stirrer glands and valves in fermenter.
16) Discuss the importance of scale-up process.
17) Give short note on lactic acid fermentation.
18) Brief the note on ion-exchange chromatography.

PART - C

III. Answer any ONE from each section of the following not exceeding1500 words each (2 x 15 = 30)

19) (a) Give an elaborate note on citric acid fermentation.
(or)
(b) Discuss microbial production of antibiotics

20) (a) Give an account on aseptic operation and containment s of a fermenter (or)

(b) Discuss the microbial growth in batch and continuous system of fermentation.
